THE EFFECT OF EPISODIC STREAM ACIDIFICATION ON THE SOUTHERN APPALACHIAN BROOK TROUT (*SALVELINUS FONTINALIS)*

A Thesis
by
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Abstract

THE EFFECT OF EPISODIC STREAM ACIDIFICATION ON THE SOUTHERN APPALACHIAN BROOK TROUT (*SALVELINUS FONTINALIS)*

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The Southern Appalachian Brook Trout (*Salvelinus fontinalis*) are currently undergoing declines in their native ranges; major causes of these declines are episodic stream acidification events. The habitats in which these animals live can regularly experience pH drops of up to 0.5-2.0 units (Neff, Schwartz, Henry, Robinson, Moore, & Kulp, 2009). When environmental pH drops this low, organisms must prevent these acids from affecting their systemic pH. The primary method of accomplishing this is through the use of the ion transporting mechanisms (Claiborne, Edwards, & Morrison-Shetlar, 2002). Previous studies on the Rainbow Trout (*Oncorhynchus mykiss*) have shown the H^+-ATPase and NHE3 to increase expression when exposed to low environmental pH (Perry, Shahsavarani, Georgalis, Bayaa, Furimsky, & Thomas, 2003). It was hypothesized that these ion transporters would be used when Southern Appalachian Brook Trout were exposed to a lowered environmental pH and that the stream sodium level would dictate which transporter was most highly expressed. To date, we have shown the presence of the H^+-ATPase and NHE3

transporters in the gills of the Southern Appalachian Brook Trout, as well as suggested that these animals change use of transporter based on current energy level and sodium content of the stream.

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This project would have never been possible without the tireless input and guidance from Dr. Susan Edwards. It is her leadership and expertise that led me to be a laboratory scientist. I would also like to thank the other members of my thesis committee, Dr. Gary Walker and Mr. Matt Kulp, for their input and commitment to the project.

A great deal of thanks must go the National Parks Service Staff, particularly Mr. Matt Kulp, in the Great Smoky Mountains National Park for their assistance with fish collections and information on the fisheries in the park. Without their passion to maintain healthy and natural fish populations in the park, this work would not have been possible.

I would like to thank my parents, Dan and Tina Moore, and my grandparents, Judson and Betty Citty and Bobby and Jane Moore, for their continued support both present and past. Their belief in my abilities was truly guided by love and faith.

Finally, I would like to thank my friends, specifically Andrew Capets, Benjamin Coe, and Scott Williams for their help with this project. They were present whenever I called, eager to assist in any way possible. Without their hours of hard work and assistance this project would not have been completed.

Dedication

I would like to dedicate this thesis and the years of work that went into it to my family and closest of friends.

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Introduction

Southern Appalachian Brook Trout

The Southern Appalachian Brook Trout (*Salvelinus fontinalis*) is the only native Salmonid to the southeastern United States. The native range of the Brook Trout stretches from Georgia to Canada, along the Appalachian mountain range, where it resides in the headwater streams of the Mississippi and Atlantic drainages (Stauffer & King, 2014). This current range of the Brook Trout has been created by a variety of geologic, geographic, and anthropogenic influences on the species (Image 1). Glacial impoundment following the ten to twelve glaciations over the past 2.5 million years and stream captures have led to many populations being isolated to cold-water lakes and high-altitude, remote streams throughout the Appalachian mountains (Karas, 1997; Galbreath, Adams, Guffey, Moore, & West, 2001). Many events of anthropogenic origin have created challenges for the species including habitat loss and degradation, competition with non-native fish, climate change, and atmospheric acid deposition (Galbreath et al., 2001; Neff et al., 2009; Neff, Schwartz, Moore, & Kulp, 2013; Robison, Scanlon, Cosby, Webb, & Galloway, 2013; Aunins, Petty, King, Schilz, & Mazik, 2014). Some declines of Southern Appalachian Brook Trout populations can be attributed to deforestation of their native habitats by timber companies. The lack of forest decreased stream quality due to sediment and runoff flowing into the streams with the absence of a riparian buffer to control it (Karas, 1997). The removal of forest canopy caused water temperature to rise, forcing fish populations to retreat to higher elevations with cooler habitats or die (Aunins et al., 2014). Timber companies, seeing the damage they had caused, looked to remediate these impoverished streams by restocking with large populations of

Northern Brook Trout as well as Rainbow and Brown trout (Karas,1997). In streams where introduced species were present, it was observed that the Southern Brook Trout fared the worst due to competition, leading to the demise of Brook Trout in many streams (Galbreath et al., 2001). The Southern Brook Trout faced competition for so long that in some areas they only persevered in physically isolated stream reaches, such as those above large waterfalls or impassable cascades. The isolation of this species to small, ecologically vulnerable habitats in headwaters and its susceptibility to minute environmental changes have resulted in the classification of the Southern Appalachian Brook Trout as a keystone species for the environments which it inhabits (King, Lubinski, Burnham-Curtis, Scott, & Morgan, 2012; Aunins et al., 2014).

Great Smoky Mountains National Park

High altitude, cold headwater habitats supporting Southern Appalachian Brook Trout are rare throughout the southern Appalachians, but a protected sanctuary exists within the Great Smoky Mountains National Park (GSMNP), straddling the North Carolina, Tennessee border (Image 2). The park boasts a wide range of habitats for organisms, especially those in aquatic environments, with habitats ranging from high altitude (2000m) to relatively low lying areas (380m) and contains approximately 4640 km of streams, all with the capability of supporting healthy trout populations (Schwartz, Cai, Neff, Rolison, & Pobst, 2011).

Within the GSMNP, water chemistry can vary dramatically between subwatersheds and even individual streams (Schwartz et al., 2011; Neff et al., 2013). This difference in water chemistry around the park can create difficulties when completing water chemistry assessments. The significance of the problem with water quality variations in the park is

highlighted upon examination of state-required water quality standards laid out in the "Clean Water Act" (United States Fish and Wildlife Service, 2002). This law requires that each state have specific water quality standards that it must meet for streams, lakes, and rivers. In North Carolina, the standards say that pH may not fall below a pH of 6 or remediation is required. In Tennessee, the same law applies but with an addendum that pH may not fluctuate more than 1.0 unit every 24 hours (NC Division of Water Quality "Red Book," Section .0200 15A: NCAC 02B .0212, 3(B); and Rules of the Tennessee Department of Environment and Conservation, Rule 0400-40-03-.03, 3(B)) (North Carolina Department of Environment and Natural Resources, 2003; Tennessee Department of Environment and Conservation, 2013). The water quality of streams and rivers that Southern Appalachian Brook Trout inhabit inside of the park regularly fall outside of the standards set by the "Clean Water Act" following storm events, putting these fish at risk of acidosis (Schwartz et al., 2011). Southern Appalachian Brook Trout populations are found in areas of the park with a pattern of high elevation, small basin watersheds which result in a lower stream Na^+ content, low acid-neutralizing capabilities of the soil, and high water flow in storm events (Neff et al., 2013).

Atmospheric Acid Deposition and Episodic Stream Acidification

Within the Great Smoky Mountains National Park, it is believed that the episodic acidification of streams associated with atmospheric acid deposition is presently the greatest factor contributing to declines of Southern Appalachian Brook Trout (Deyton, Schwartz, Robinson, Neff, Moore, & Kulp, 2009; Neff et al., 2009; Wesner, Cornelison, Dankmeyer, Galbreath, & Martin, 2011). Atmospheric acid deposition is a phenomenon that occurs due

to poor industrial and agricultural practices in the late 18th century until present (Driscoll, Driscoll, Mitchell, & Raynal, 2003). Increased acidic emissions were caused beginning with the industrial revolution with the burning of fossil fuels that release SO_2, NO, NO_2, and NH_3 into the atmosphere. These gasses attach to water vapor and fall with precipitation in the form of H_2SO_4, HNO_3, and NH_4^+ (Driscoll et al., 2003).

The Great Smoky Mountains National Park receives elevated rates of atmospheric acid deposition in comparison with other areas on the east coast, resulting in increased episodic stream acidification events, adding to the acidification of soil and surface waters in the park (Neff et al., 2009). Episodic Stream acidification occurs when increased rates of atmospheric acid deposition occur, which lower stream pH and increase stream gauge (Deyton et al., 2009; Neff et al., 2009). These periods of increased flow and decreased pH are usually short and acute, resulting in the decreased ability for surrounding soils effectively to buffer acids leaching into the streams (measured as acid-neutralizing capability) leading to severe pH fluctuations (Deyton et al., 2009).

The acid-neutralizing capability of an area depends on the geographic, geologic, and ecological properties of that environment (Deyton et al., 2009). High levels of atmospheric acid deposition in an area reduce the buffering capacity of the soils in the area (Petty & Thorne, 2005; Deyton et al., 2009). High-elevation, small area, steep-sloped watersheds (like the headwater streams where Brook Trout tend to reside) are particularly susceptible to episodic stream acidification resulting from the inability of precipitation, flowing at high velocities down the slopes, to buffer through the soil before entering streams (Neff et al., 2013). Organic acids also contribute to the acidification of streams in the form of natural plant nitrates carried from surface soils into stream channels (Deyton et al., 2009).

The direct effect of acid deposition on stream pH is not the only impact to fish health caused by episodic stream acidification events. Constant recursions of these events are known to diminish populations of aquatic invertebrates, the primary diet of Brook Trout, to only a few acidophilic species (Petty & Thorne, 2005). Large storm flow events are also known to strip watersheds of excess ions. This trend is compounded in streams with traditionally low ion concentrations, especially Na^+ (Cai, Schwartz, Robinson, Moore, & Kulp, 2011; Neff et al., 2013). During these events, trout are not able to meet their Na^+ needs through the Sodium-Hydrogen Exchanger (NHE), which increases whole body H^+ concentrations, requiring the activation of the energetically costly proton pump (H^+-ATPase), and general physiological distress (Neff et al., 2009).

Acid/Base Regulation

All aquatic vertebrates must maintain internal pH homeostasis to prevent naturally occurring acids in the environment from building up and affecting internal processes (Claiborne et al., 2002). Fish are highly sensitive to even minute changes in environmental pH which can alter blood pH, resulting in changes in enzyme function, disturbance of ion regulation mechanisms, circulatory collapse, and in extreme cases death (Claiborne et al., 2002; Evans, Piermarini, & Choe, 2005; Neff et al., 2009).

Living in an aquatic environment forces fish to utilize other methods of acid-base ion regulation compared to those used by terrestrial organisms. Maintaining internal ionic homeostasis in fish is achieved by buffering mechanisms, such as bicarbonate or other "non-bicarbonate" buffers, and more effectively by ion transport mechanisms (Claiborne et al., 2002). Non-bicarbonate buffers (Nbbs) can use a variety of ions to control the amount of free

H^+ in the intracellular fluids of a fish; examples include erythrocytic hemoglobin and specific types of phosphates (Claiborne et al., 2002). The variety of Nbbs allows for homeostatic regulation of both acidic and basic intracellular fluid fluctuations, but the low quantity of these ions available in blood plasma limits the effectiveness of this system (Claiborne et al., 2002). Bicarbonate buffering uses a bicarbonate molecule (HCO_3^-) to combine with excess H^+ in the plasma of fish which results in forming new CO_2 and H_2O molecules, known as the Henderson-Hasselbalch equation ($CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$) (Claiborne et al., 2002). The CO_2 produced from this reaction can be diffused across the gill epithelium, but when production of CO_2 is greater than diffusion rates, a buildup occurs in the blood, which causes the blood to become acidic (Evans et al., 2005). This situation is easily solved in mammals by increasing ventilation rates, expelling all excess CO_2 in the bloodstream. The ease of CO_2 excretion is due to the high plasma partial pressure of CO_2 (notated PCO_2) in mammals (Claiborne et al., 2002). Aquatic organisms such as fish have a much lower PCO_2 than mammals with their environment; this, along with the high solubility of CO_2 in water (compared to oxygen), creates a very low CO_2 pressure gradient between the internal and external environment, making diffusion more difficult (Claiborne et al., 2002). The limited ability for fish to manage CO_2 levels by hyperventilation limits the effectiveness of bicarbonate buffers in aquatic mediums, forcing these organisms to predominantly rely on ion transfer mechanisms to maintain homeostatic conditions. The transfer of acid-base relevant ions from the circulatory system of the fish to the external environment is accomplished through a variety of paths: kidneys, gills, intestine, skin, and bladder have all been shown to play a role in effectively reducing internal pH of freshwater fish (Evans, Piermarini, & Potts, 1999; Evans et al., 2005).

The regulation of pH is not the only challenge for freshwater fish. Because of the low sodium content of freshwater environments they must maintain a hyperosmotic state compared to the external environment. Maintaining a specific hyperosmotic ion concentration prevents the fish from becoming too salt-depleted or overhydrated (Evans et al., 2005; Evans, 2008). This results in a hyperosmotic state in freshwater fish in which the animal is constantly loosing salts (NaCl) to their external environment and gaining water (Image 3). To counter this, animals must actively uptake salts from the environment and excrete copious amounts of dilute urine. Ion balance and pH regulation are intrinsically linked. Research has shown the coupling of Cl^- with HCO_3^- and OH^-, and Na^+ with NH_4^+ and H^+ provide passive channels for ion regulation (Claiborne et al., 2002; Perry et al., 2003).

Two of the primary mechanisms associated with the homeostatic regulation of salinity and pH are Na^+/H^+ exchangers (NHE) and H^+-ATPases, respectfully (Image 4). These transport proteins function as their names imply; NHEs function by the electroneutral transfer of internal H^+ ions for environmental Na^+ ions via, H^+-ATPase uses metabolically produced ATP to pump H^+ across the plasma membrane of cells (Evans et al., 2005). When studying membrane transport proteins, it quickly becomes apparent that function is rarely a process of maintaining a single ion's homeostasis. These transporters work as a unit to maintain a balance between the organism and external environment. It has been shown that the outward excretion of H^+ (via H^+-ATPase) creates a favorable electrochemical gradient for the uptake of Na^+, activating passive Na^+ channels identified as ASICs (Acid Sensing Channels) (Claiborne et al., 2002; Perry et al., 2003; Dymowska, Schultz, Blair, Chamot, & Goss, 2014). Thermodynamic studies on the H^+-ATPase have shown it to be coupled with an accompanying Na^+ channel (Parks, Tresguerres, & Goss, 2008), which was hypothesized to

be ASIC by Dymowska et al. (2014) through a series of sodium flux experiments on Rainbow Trout. Channels such as ASIC are hypothesized to function on a gradient that will continue to accept ions until a homeostasis is reached, making this a valuable mechanism for Na^+ regulation when H^+-ATPase is active (Dymowska et al., 2014) (Image 5).

The Cl^-/HCO_3^- exchanger is regarded as the primary mechanism for base regulation in freshwater teleost, although it can support both acid and base regulation (Claiborne et al., 2002; Evans et al., 2005; Perry et al., 2003). The Cl^-/HCO_3^- exchanger is an electroneutral pathway and has been studied in its function with H^+-ATPase because of the low electrochemical gradient it naturally exhibits (Claiborne et al., 2002; Perry et al., 2003). Studies have suggested that for a Cl^-/HCO_3^- exchange to become active, an excess of intracellular HCO_3^- must be created by H^+-ATPase, linking the two mechanisms in regulation (Piermarini & Evans, 2001; Perry et al., 2003). The function of a Cl^-/ HCO_3^- exchanger in acidosis is limited to allow for a buildup of HCO_3^- to assist in pH management; and in alkalosis, the function is increased to rid intracellular fluids of HCO_3^-, allowing pH to balance (Perry et al., 2003). The activity state of all the aforementioned ion transporters constantly fluctuates due to environmental water chemistry. This makes the process of ionic and osmotic balance a coordinated orchestration of upregulation and downregulation of transport proteins to keep the organism in a homeostatic state.

Structure and Function of H^+-ATPase

The H^+-ATPase is an energy-dependent transport protein that uses cellular energy in the form of ATP to transport protons across the plasma membrane, effectively lowering internal pH and maintaining ionic homeostasis (Nishi & Forgac, 2002; Evans et al., 2005).

H^+-ATPase is structured as a dual-domain, multi-subunit rotary proton pump system (Marshansky & Futai, 2008). The first domain, V_1, is comprised of eight subunits (A_3, B_3, C, D, E, F, G_2, H_{1-2}) which are responsible for ATP hydrolysis, creating energy to power the second, V_0, domain (Marshansky & Futai, 2008) (Image 6). The V_0 domain contains six subunits (a, c_4, c', c", d, e), which function by transporting protons across the plasma membrane through the use of H^+-ATPase's rotary mechanism (Marshansky & Futai, 2008). The rotary mechanism is able to function by its attachment to the stalk (subunit D), which can turn when powered by the energy created during ATP hydrolysis in V_1 (Marshansky & Futai, 2008). This pathway is heavily ATP dependent; therefore, it represents a high metabolic cost for any organism in which it is active. While functioning, the H^+-ATPase uses one ATP for the transfer of two to four H^+ across the plasma membrane of the cell (Maxson & Grinstein, 2014). The V_1 domain allows for the attachment of three ATP at once, making the transfer of six to twelve H^+ possible every cycle of the V_0 domain (Maxson & Grinstein, 2014). Being able to transfer large numbers of H^+ across the plasma membrane in a relatively short period of time makes the H^+-ATPase a very valuable transport protein for freshwater organisms, even at its high metabolic cost. The high metabolic cost of this transporter limits the other physiological process that can be active at the same time, taking energy away from reproductive, maturation, and muscular efforts. This pathway is thought to be the primary mechanism for acid excretion and pH regulation in freshwater teleost (Evans et al., 2005).

Structure and function of the Na^+/H^+ Exchanger

The Na^+/H^+ Exchangers (NHE) are a family of membrane transport proteins that function via an inward Na^+ gradient and an outward H^+ gradient (Claiborne et al., 2002; Evans et al., 2005). The goal of NHE function is to maintain an ionic homeostasis, electroneutrally exchanging one external Na^+ ion for and internal H^+, as well as to regulate internal pH and cell volume (Claiborne et al., 2002). The NHE family of molecules was at first thought to be limited to saltwater adapted teleosts, but more recent information has shown its presence in freshwater teleosts (Claiborne et al., 2002). To date, there are nine proposed isoforms of NHE described for both freshwater and saltwater adapted fish in the literature (NHE1-NHE9) of which most are similar in structure and function (Edwards, Tse, & Toop, 1999; Fliegel, 2005). Each NHE isoform is identified by two domains: an integral membrane domain ("N"-terminus) and a cytosolic tail domain ("C"-terminus) (Fliegel, 2005) (Image 7). The 5' N-terminus consists of 10-12 membrane-spanning domains; and the C-terminus is specific to each isoform, and for this reason, it is thought to play a regulatory role.

Gill Morphology of Freshwater Teleosts

The brachial region of freshwater teleosts is comprised of four pairs of gill arches, each of which is covered in smaller gill filaments, all protected by the operculum, a bony flap that covers the gills (Evans et al., 2005) (Image 8). The gills function by circulating blood counter-current to water flow across the gill epithelium, maximizing the ability for diffusion across the epithelium. Fish can gain dissolved oxygen and other ions from the surrounding environment by pulling water into the pharynx and over the gill epithelium, allowing for

diffusion and ion transfer; then, water can exit through the operculum (Evans et al., 2005). The gill epithelium is highly folded for increased surface area and has a complex vasculaculture, allowing large amounts of blood to be cycled through the gill filaments where cells with the responsibility of ion regulation and gas exchange processes reside (Evans et al., 2005). Epithelial tissues across the gill surface are covered with two primary cell types, pavement cells (PVC) and mitochondrial rich cells (MRC) (Evans et al., 2005). PVCs are thin, cuboidal cells located throughout the gill filament and interlamellar region typically associated with sites of gas exchange. MRCs are found in the interlamellar region and trailing edge of gill filaments where they exist as large, oval cells that have a distinct polarity associated with them (Evans et al., 2005). MRCs are aptly named, as they are dense with mitochondria, making great vessels for active transporters. This differs from PVC's, which have lesser densities of mitochondria (the reason they were previously considered to play a passive role in physiological processes) (Evans et al., 2005). PVCs are densely packed in the gill epithelium covering greater than 90% of the surface, whereas MRCs tend to be scattered through the dense PVC stratum individually, covering less than 10% of gill surface area (Evans et al., 2005) (Image 9 & 10).

Sub-populations of MRCs and PVCs have been described, as well as the independent functions of each in osmoregulatory processes. Peanut lectin agglutinin (PNA) has been used to describe sub-populations of these cells, as it shows a positive binding preference to cells closely related to MRCs. Analysis of PNA's specific binding preference shows specific differences in standard MRCs (positive binding, identified as β-MRC) and Mitochondrial-rich PVCs (no binding, identified as MR-PVC or α-MRC) (Goss, Adamia, & Galvez, 2001). The function of these two sub-populations of MRC have been described in Rainbow Trout by

exposing the fish to hypercapnic conditions, effectively lowering blood pH (Galvez, Reid, Hawkings, & Goss, 2002). Under these conditions α-MRCs exhibited an increased expression of H^+-ATPase as opposed to β-MRCs, showing an association of this transporter to the α-MRC sub-population (Galvez et al., 2002). MRC research in other anatomical epithelia (vertebrate kidney) has identified functional ion differences in the α-MRC and β-MRC, showing the α-subpopulation associated with acid excretion and the β-subpopulation associated with base secretion (Perry et al., 2003). This idea has been adopted to apply to the gill epithelium of all freshwater teleost, linking the H^+-ATPase and NHE transporters to the α-MRC (Perry et al., 2003).

Previous Work on Project

Previous work in Dr. Susan Edwards research lab by Brian Mikeworth focused on the location of H^+-ATPase and NHE in the gills of the Southern Appalachian Brook Trout. This research was able to localize H^+-ATPase, NHE2, and NHE3 to the MRCs along the apical surface of the gill lamellae and the interlamellar region of the gill filament through immunohistochemical procedures. Another aspect of this project that was used in developing the background and methodology for my current project was a comparative expression analysis of H^+-ATPase and NHE3 gill protein following exposure to an acidified environment while looking at fish from three different locations along a stream gradient. Fish were taken from a high, middle, and low altitude point along the same stream and western blotting techniques were used to quantify gill protein expression. The only statistically significant data found in this analysis was a decrease in H^+-ATPase gill protein expression from the middle altitude to low altitude sample sites. The identification of H^+-

ATPase and NHE3 in the gill of the Southern Appalachian Brook Trout by Brian Mikeworth set the foundation for the research presented here.

Purpose of Study

The purpose of this study is to determine if, and to what level, Southern Appalachian Brook Trout respond to the episodic acidification of streams using the Na^+/H^+ Exchanger (NHE3) and H^+-ATPase. The episodic acidification of streams is known to strip streams of Na^+ ions and greatly reduce environmental pH (Cai et al., 2011; Neff et al., 2013). These environmental conditions can lead to decreases in whole body sodium and blood pH in freshwater organisms, which can lead to sub-lethal and possibly lethal stress in these organisms. NHEs function to rid fish of excess H^+ in environments abundant in sodium ions. In environments with a relatively low content of sodium ions, the H^+-ATPase is thought to maintain an internal pH homeostasis.

The hypothesis presented here is that in lower sodium content freshwater streams, when exposed to low environmental pH conditions, the H^+-ATPase transporter is the leading mechanism associated with acid-base regulation in the Southern Appalachian Brook Trout. Likewise, in higher sodium content freshwater streams, the Na^+/H^+ exchanger plays a major, if not primary role in acid-base regulation during episodic stream acidification events. To determine the validity of this idea, molecular biology techniques and experimentation were conducted on the Southern Appalachian Brook Trout in a laboratory setting to look at NHE3 and H^+-ATPase in the gills of this organism.

Methods

Collection of Samples

Southern Appalachian Brook Trout were collected from two locations in the Great Smoky Mountains National Park on two different dates (Image 2). Southern Appalachian Brook Trout (*Salvelinus fontinalis*) were obtained via standard backpack electroshocking techniques with the assistance of the National Parks Service. On the first sampling date, in October of 2012, 28 fish were obtained from Road Prong on the Tennessee slope of the park for the constant sodium experiment. The second sampling date, in March of 2015, 21 fish from each stream (42 total, used for the sodium alteration experiment) were obtained from Road Prong (low salinity sample) and Beech Flats Prong (high salinity sample), which straddle the Tennessee and North Carolina sides of the park respectfully. Fish were held in large holding tanks until experimentation: 30 days for experimentation 1 and 15 days for experimentation 2. The water chemistry for each stream was taken in the field and when the fish were introduced to the laboratory stream. Salinity, temperature, and pH for Beech Flats (high sodium sample) were recorded at 0.02ppt, 10.8°C, and 7.08 in the field, respectfully; and 0.06ppt, 9.4°C, and 8.04 for the laboratory stream. Salinity, temperature, and pH for Road Prong (low sodium sample) were recorded at 0.01ppt, 5.4°C, and 7.46 in the field, respectfully; and 0.04ppm, 8.7°C, and 7.97 for the laboratory stream.

Experimental Design and Sacrificing

Fish were exposed to either a control or experimental treatment, or a zero time point sacrifice, during the course of this project. Experimental fish were transferred from a holding

tank to an experimental tank containing deionized water lowered to a pH of 4.8 (diluted by HCl) for a set time period (2, 4, and 6 hours for the first experiment; 30 mins, 1 hour, and 2 hours for the second). Control fish were transferred into tanks containing deionized water at a pH of 7.4 and left for the same period of time as the corresponding experimental treatment. Post experimentation, fish were anesthetized by MS-222 (0.05%). Tissues (gill, kidney, and skin) were removed for further experimentation and either flash-frozen in liquid nitrogen or fixed in 4% paraformaldehyde. To determine if blood pH and fish weight were correlated, blood was drawn and pH was measured for each fish sampled in the constant sodium and sodium alteration experiments. Zero time point fish were sacrificed from the holding tank with no treatment.

RNA Isolation and cDNA Synthesis

Flash frozen tissues were homogenized in Tri-Reagent (Sigma-Aldrich, St. Louis, MO) to extract RNA from the soft tissues; all parts of the mixture were centrifuged to separate contents by density; and the process was completed based on the manufacturers protocol. RNA was solubilized from pellet form in RNA/DNAse free water and quantified via spectrophotometer at 260/280 nm (NanoDrop 2000, Thermo Fischer Scientific, Waltham, MA). First Strand complementary DNA (cDNA) was synthesized from 5μg of total RNA using a Superscript III reverse transcriptase enzyme and Oligo-dT Primers (Invitrogen, Carlsbad, CA).

Polymerase chain reaction (PCR)

H^+ATPase. A set of primers based upon cDNA sequenced by Perry et al. (2000) for Rainbow Trout (*Oncorhynchus mykiss*) were designed for experimentation. A polymerase chain reaction (PCR) procedure was performed (10x PCR Buffer, 50mM MgCl, 10mM dNTP mix, 10µM Forward primer, 10µM reverse primer, template cDNA, and Platinum TAQ polymerase (Invitrogen, Carlsbad, CA)). A Bio-Rad MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) was used for the execution of the thermocycling program (94°C (2 minutes), 94°C (30 seconds), gradient of 54°C-58°C (30 seconds), 72°C (45 seconds) was cycled 35 times, with a final extension time of 72°C (10 minutes)). Gel electrophoresis was used to analyze the PCR products using an agarose gel (1% agarose in 1x TBE Buffer) and ethidium bromide staining to allow viewing under UV light. An AlphaImager HP System (Protein Simple, San Jose, CA) was used to image the gel. Optimum annealing temperature for this primer set was found to be 57.7°C.

NHE3. A set of primers were designed based on the DNHE3 sequence outlined in Ivanis et al. (2008). A polymerase chain reaction procedure was preformed (10x PCR Buffer, 50mM MgCl, 10mM dNTP mix, 10µM Forward primer, 10µM reverse primer, template cDNA, and Platinum TAQ polymerase (Invitrogen, Carlsbad, CA)). A Bio-Rad MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) was used for the execution of the thermocycling program (92°C (2 minutes), 92°C (30 seconds), gradient of 54°C-60°C (45 seconds), 72°C (45 seconds) was cycled 40 times, with a final extension time of 72°C (10 minutes). Gel electrophoresis was used to analyze the PCR products using an agarose gel (1% agarose in 1x TBE Buffer) and ethidium bromide staining to allow viewing under UV

light. An AlphaImager HP System (Protein Simple, San Jose, CA) was used to image the gel. Optimum annealing temperature for this primer set was found to be 54°C.

Molecular cloning and sequencing

Cloning and sequencing was performed using pGEM-T Easy Vector System (Promega, Madison, WI). PCR products corresponding to the expected size were ligated into pGEM-T easy vector and transformed to JM109 High Efficiency Competent Cells (Promega, Madison, WI). The transformed cells were plated onto premade LB-AMP plates (Teknova, Hollister, CA) and grown overnight at 37°C. Successfully grown colonies were picked with a sterilized pipette tip and placed in LB-AMP broth (2% LB broth with 0.1% Ampicillin added), vortexed, and left on a shaker (150 rpm) at 37°C overnight. A FastPlasmid miniprep kit (5 Prime Inc., Gaithersburg, MD) was used to isolate plasmid DNA before spectrophotometer quantification at 260/280nm (Nanodrop 2000, Thermo Fischer Scientific, Waltham, MA). A restriction digest using EcoR1 was used to analyze the plasmid DNA for the presence of product insert. Plasmid clones of the containing the wanted insert were sent for sequencing to Mount Desert Island Biological Laboratory (Salisbury Cove, ME) on an ABI PRISIM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Quantative Real Time - Polymerase Chain Reaction (qRT-PCR)

Relative expression of H^+-ATPase was quantified using primers previously designed for the experimentation. The L8 protein was used as an endogenous control for the comparative C_T ($\Delta\Delta C_T$) method on the 7500 Real Time PCR System (Applied Biosystems, Carlsbad, CA). Primer and cDNA concentrations were optimized for the experimentation.

Optimization for H^+-ATPase resulted in concentrations of 50ng of template cDNA, 50ng of H^+-ATPase forward and reverse primer, and 300ng of L8 forward and reverse primer. These ingredients as well as 0.5µg of ROX reference dye diluted 1:10 with sdH_20, and SYBR GreenER SuperMix (Invitrogen, Carlsbad, CA) were mixed into a master-mix before the template cDNA was added for a total of 25µl per well. The expression of mRNA in experimental individuals was normalized with control individuals through the endogenous control. Melt curve analysis confirmed the amplification of the desired product through the 7500 software (Applied Biosystems, Carlsbad, CA). The C_t value (Threshold value) and RQ (Relative Quantification) is determined through the 7500 software.

Antibodies

H^+-ATPase. A monoclonal mouse anti-H^+-ATPase heterologous antibody was used to identify H^+-ATPase protein expression (V-ATPase 60kDA subunit B in yeast; purchased from Invitrogen, Carlsbad, CA). This antibody has been shown successful at identification of H^+-ATPase 60-kDa subunit B in yeast (Kane, Kuehn, Howald-Stevenson, & Stevens, 1992). This antibody was used for previous experiments in Dr. Susan Edwards' research lab.

NHE3. The NHE3 antibody used in experimentation was a gift from Dr. Steven F. Perry; University of Ottawa, Canada. This polyclonal antibody was raised against Rainbow Trout (*Oncorhynchus mykiss*) in a rabbit NHE3 (ETKADVDFNKKFRAAS, ABO32815) and was used to identify NHE3 protein expression. The NHE3 antibody has validated in studies on the Rainbow Trout (Ivanis, Esbaugh, & Perry, 2008).

Protein Extraction and Expression Quantification

Flash Frozen tissue samples were homogenized in buffer on ice (0.18g Tris-Base, 4.28g sucrose, 0.5ml 100mM EDTA, pH 7.4) allowing for separation of parts by centrifugation. Protein content was determined through BCA protein assay (Thermo Fischer Scientific, Waltham, MA). Total protein samples (20μg) were loaded in Criterion TGX gels (Bio-Rad, Hercules, CA) and separated by SDS-PAGE. Proteins in gel were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) via electrophoresis (20 minutes at 50v, 25 minutes at 75v, and 40 minutes at 150v). Membranes were blocked in a 5% blotto solution (5% nonfat dry milk powder, 0.1M Tris-buffered saline with 0.2% Tween-20 (TBST) for 2 hours at 4°C on a rocker. Membranes were then incubated in 5% blotto with primary antibody (H^+-ATPase 1:500; NHE3 1:1000) on a rocker overnight at room temperature. Blots underwent 3 washes in 1x TBST, followed by incubation in horseradish peroxidase (HRP) conjugated secondary antibody (Goat anti-Mouse HRP 1:20,000; NHE3 1:10,000) with Precision Protein StrepTactin-HRP(1:15,000) conjugate (Bio-Rad, Hercules, CA) in 1xTBST for 1 hour at room temperature. Excess secondary antibody solution was washed from the membranes by 3 washes in 0.1M TBS. Membranes were prepared for analysis using a chemiluminescent substrate, Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and imaged on a Bio-Rad ChemiDoc Imager (Bio-Rad, Hercules, CA). Images were analyzed using Image Lab Software (Bio-Rad, Hercules, CA) to determine relative protein expression.

Statistical Analyses

Statistical significance was determined through multiple analyses and software programs, all noting significance at p=.05. Trends in the blood pH, fish weight, and plasma osmolarity were determined through correlation analysis in Microsoft Excel. Significance in the blood pH, fish weight, and plasma osmolarity relationships were determined through regression analysis and an Analysis of Covariation (ANCOVA) in Microsoft Excel and SPSS (IBM; Armonk, New York), respectfully. Significance between individual treatment pairs was determined through t-test in Microsoft Excel. Data graphed for t-test results are presented as data mean ± SEM (one standard error of mean). The effect of treatment, time, and interactions of the two were determined through a two-way Analysis of Variance (ANOVA) in Microsoft Excel.

Results

Molecular Identification

H^+-ATPase gill mRNA expression. Previous work by Mikeworth (2012) identified an Open Reading Frame (ORF) of 2093bp with a shared homology of 87.5% with Rainbow Trout and 86.9% with Zebrafish.

NHE3 gill mRNA expression. RT-PCR of NHE3 yielded PCR products of 1167bp and when sequenced were confirmed to share a 97% homology with Rainbow Trout NHE3b (NP 001153954.1) (Figure 1).

Blood pH and Osmolarity Analysis

Constant sodium experiment. Data from the constant sodium experiment did not show a strong correlation (R^2=.091) between blood pH and fish weight amongst the 28 fish sampled. When split into treatment groups, the lack of correlation was retained (Control R^2=.002, Experimental R^2=.258) (Figure 2). Regression analyses of the data were found to be insignificant. An ANCOVA of the data found no significant effect of fish weight or treatment on blood pH.

Sodium alteration experiment. The sodium alteration experiment showed no correlation (R^2= .206) between blood pH and fish weight for the 42 fish sampled. When split into treatment groups, the lack of correlation was retained for a relationship between fish weight and blood pH (Control R^2=.04, Experimental R^2=.12) (Figure 3). Regression Analyses of the data was found to be insignificant.

Blood plasma osmolarity was measured for each fish in both the control and experimental condition. No correlation was seen for the comparison of blood pH and plasma osmolarity (All Data R^2=.080, Control R^2<.001, Experimental R^2=.003) (Figure 4). Regression Analyses of the data were found to be insignificant.

An ANCOVA of the low salinity stream data found no significant effect of fish weight or treatment on blood pH nor an effect of treatment or plasma osmolarity on blood pH. An ANCOVA of the high salinity stream (using fish weight, treatment, and blood pH as variables) found a significant effect of treatment on blood pH, $F(1,15) = 5.91$, p=.03, but no effect of weight. An ANCOVA of the high salinity stream (using treatment, plasma osmolarity, and blood pH as variables) found a significant effect of treatment on blood pH, $F(1,15) = 4.66$, p=.05, but no effect of plasma osmolarity.

Quantitative Real-Time PCR

H^+-ATPase gill mRNA expression.

Constant sodium experiment. There were no significant differences in H^+-ATPase mRNA expression between group means at any time point or in the control (Figure 5). One outlying value has affected the 4-hour experimental value, but the mRNA expression is constant across all values otherwise. A two-way ANOVA of the H^+-ATPase gill protein data found no significant effect of treatment, time, or an interaction of the two.

Sodium alteration experiment. The H^+-ATPase mRNA expression in the sodium alteration experiment showed no significant differences between group means at any time point or in the control and was relatively constant across all time points for both the high and low sodium streams (Figure 6). A two-way ANOVA of the H^+-ATPase gill protein data

from the high and low sodium streams found no significant effect of treatment, time, or an interaction of the two.

Protein Quantification

H^+-ATPase protein expression in gill tissue. The H^+-ATPase antibody was used to determine total protein expression in Southern Appalachian Brook Trout gill tissue. Western blots identified a single immunoreactive band at 65kDa, similar to previously reported studies (Perry et al. 2000).

Constant sodium experiment. H^+-ATPase protein expression showed no significant differences between group means at any time point or in the control (Figure 7). Though non-significant, the experimental values appear to have an increasing trend, while the control values are constant; if this trend is present, it could be teased out by extending time points. A two-way ANOVA found no significant effect of treatment, time, or an interaction of the two.

Sodium alteration experiment. During the sodium alteration experiment fish from the low sodium stream showed significant (p=.012) upregulation of H^+-ATPase gill protein expression at 30 minutes when means were compared with a t-test (Figure 8). A decreasing trend was seen through the low sodium experimental samples as time increased. A two-way ANOVA of the H^+-ATPase gill protein data from the low sodium stream found a significant effect of treatment, $F(1,12) = 21.22$, $p<.01$; time and interaction were not found to be significant factors.

Analysis of the high sodium stream H^+-ATPase gill protein data did not reveal any statistical significance when means were compared with a t-test (Figure 8). A two-way ANOVA of the H^+-ATPase gill protein data from the high sodium stream found a significant

effect of treatment, $F(1,12) = 9.35$, $p=.01$, time and interaction were not found to be significant factors.

NHE3 expression in gill tissue. Results using the Rainbow Trout NHE3 antibody demonstrated two immunoreactive bands in western blots for Southern Appalachian Brook Trout gill tissue (one at 95kDa, the other at 97kDa) similar to results reported by Ivanis et al. (2008). Pixel density of the band located at 95kDa was used for analysis, following methods of Ivanis et al. (2008).

Sodium alteration experiment. A significant upregulation of NHE3 gill protein expression was recorded in the low sodium experimental sample at 1 hour ($p=.004$) and 2 hours ($p=.016$) when means were compared with a t-test (Figure 9). An increasing trend was seen through the low sodium experimental samples as time increased. A two-way ANOVA of the NHE3 gill protein data from the low sodium stream found a significant effect of treatment, $F(1,12) = 27.02$, $p<.01$, and a significant interaction $F(2,12) = 5.27$, $p=.02$. Time was not found to be a significant factor through the use of a two-way ANOVA for analysis.

No statistical significance was recorded for the high sodium stream NHE3 gill protein samples when means were compared with a t-test (Figure 9). A two-way ANOVA of the NHE3 gill protein data from the high sodium stream found no significant effect of treatment, time, or an interaction of the two.

Discussion

The initial goal of this study was to determine if H^+-ATPase is upregulated in gill tissue when Southern Appalachian Brook Trout are submitted to acidic conditions, but the research led to further questions. To address these questions, the goal became to examine if transport proteins were expressed differentially following alteration of environmental sodium concentrations. A two-part hypothesis was developed to address all questions: 1) Southern Appalachian Brook Trout collected from a low environmental sodium stream will utilize the H^+-ATPase transporter to maintain acid-base homeostasis when exposed to a low environmental pH, and 2) Southern Appalachian Brook Trout collected from a high environmental sodium stream will utilize the NHE3 transporter to maintain acid-base homeostasis when exposed to a low environmental pH. These hypotheses were based on current literature on NHE3 and H^+-ATPase in the gills of Rainbow Trout (Claiborne et al., 2002; Perry et al., 2003; Evans et al., 2005; Ivanis et al, 2008).

Blood pH and Plasma Osmolarity

Comparing the values of blood pH, plasma osmolarity, and fish weight, gives an idea of the correlation of these values. In preliminary discussions on the experiment, the idea that larger fish may be able to buffer acids better due to their size was considered, it was found that this was not the case. In the second set of experiments (Sodium Alteration Experiment), it was decided to add a measurement of plasma osmolarity as well. Blood pH and plasma osmolarity are related by the tonicity of the blood, cells swell and shrink due to changes plasma osmolarity. The levels of bicarbonate determine the osmolarity and nonbicarbonate

buffers present in the system, which are determined by pH (Heming, Boyarsky, Tuazon, Bidani, Thomas, & Divina, 2000). The extraction and measurement of blood was to determine if environmental acidification had caused any changes in blood pH. The stability of the blood pH between control and experimental fish suggested that the membrane ion transporters were active, preventing blood pH from dropping to environmental pH levels.

Looking at relationships of this data, it was noticed that the Southern Appalachian Brook Trout keeps a standard blood pH that is slightly acidic (between 6.7-7.3). The standard blood pH of a teleost fish is around 7.0-7.4 (Claiborne et al., 2002). Whether this is a factor of environmental conditions in the GSMNP or an effect of laboratory settings is unknown. This could be further investigated with a series of environmental field tests on the fish.

Experimental Design and Sodium Levels

Experimental design called for a high and low sodium stream that matched the environmental stream conditions of the collected fish. Due to difficulties in managing the laboratory stream sodium levels, the sodium concentrations were not able to stabilize to match environmental levels 100%. Both the high and low sodium streams were slightly higher in sodium content than their respective environmental streams. The high sodium stream (Beech Flats) had an environmental salinity level of .02ppt, and the laboratory stream was held at .06ppt. The low sodium stream (Road Prong) had an environmental salinity of .01ppt, and the laboratory stream was held at .04ppt. For experimental integrity differences between sodium levels were not considered in statistical analyses.

Operation of Gill Transport Mechanisms

Previous studies have shown the use of H^+-ATPase and NHE3 as mechanisms for the regulation of pH homeostasis in freshwater teleosts (reviewed by Claiborne et al., 2002). The previous work of Brian Mikeworth (2012) used specific primers to show that Southern Appalachian Brook Trout H^+-ATPase is highly conserved between Rainbow Trout (87.5% homology) and zebrafish (86.5% homology). Work on the current project was able to show, through the use of primers designed from Ivanis et al. (2008), that Southern Appalachian Brook Trout NHE3 shows an incredibly high level of conservation with Rainbow Trout NHE (97% homology). This high degree of conservation in H^+-ATPase and NHE between Southern Appalachian Brook Trout and Rainbow Trout suggests a common origin and function of these transporters.

Gill mRNA expression. The H^+-ATPase primers were used for qRT-PCR for both the constant sodium and sodium alteration experiments. No significant gill H^+-ATPase mRNA upregulation or downregulation was seen between control and experimental treatments in the constant sodium or sodium alteration experiments. The 4-hour experimental treatment in the constant sodium experiment is the only outlier present. This increased H^+-ATPase mRNA expression is possibly due to a high variance between samples within the treatment. Other than this treatment, all other values are held relatively constant across all time points.

According to the process of transcription and translation, mRNA upregulates before the corresponding proteins. This is thought usually to occur before 2 hours but has been shown to occur even more quickly, within 15 minutes of exposure to stimuli (reviewed by Claiborne et al., 2002; Weakley, Claiborne, Hyndman, & Edwards, 2012*)*. In these

experiments, it is suggested that very early (less than 30 minutes) mRNA transcription and translation is the cause of the lack of variation in the results for qRT-PCR experiments. This very quick transcription and translation of mRNA to protein could arise from the speed in which environmental acidification episodes occur in the wild, dropping pH 0.5-2.0 units in a very short time, requiring the animals to begin using the membrane transport proteins very quickly (Neff et al., 2009).

Gill protein expression.

H^+-ATPase. Perry, Beyers, & Johnson (2000) showed a conservation of H^+-ATPases across many organismal lineages including human and marine organisms. The high H^+-ATPase homology across lineages strongly suggests that function is conserved as well, allowing the use of a commercially produced mammal H^+-ATPase antibody for protein expression experiments.

NHE3. The high conservation of NHE3 between Southern Appalachian Brook Trout and Rainbow Trout led to the use of the a Rainbow Trout NHE3 antibody designed by Ivanis et al. (2008) to show protein expression.

Implications of results. The presence of both H^+-ATPase and NHE3 transport proteins were identified through western blots by bands at 65kDa and 95kDa, respectively. These specific bands were referenced in literature and used for quantification of expression across all samples (Perry et al., 2000; Ivanis et al., 2008). The hypothesis expected to see an increase in H^+-ATPase gill protein expression in animals exposed to low sodium streams and an increase in NHE3 gill protein expression in high sodium streams under experimental conditions. However, this was not the case. During the sodium alteration experiment, experimental H^+-ATPase gill protein expression significantly increased at the 30-minute time

point in samples from the low sodium stream, following the hypothesized H^+-ATPase mRNA expression increase (at > 30 minutes). A two-way ANOVA on the H^+-ATPase data found a significant treatment effect for both the high and low sodium streams, suggesting the differential expression was a result of the decreased stream pH. When looking at the NHE3 gill protein expression, a significant upregulation was seen in the experimental treatments from the low sodium stream at the 1 hour and 2 hour time points compared to the respective control values. A two-way ANOVA on the NHE3 data found a significant treatment and interactive effect of treatment and time for the high sodium stream, suggesting that differential expression was a result of the decreased stream pH.

NHE3 requires sodium to function, and H^+-ATPase requires an abundance of ATP to function. The analysis of H^+-ATPase gill protein expression in the low sodium stream showed an increase in protein expression immediately at 30 minutes, followed by a rapid decrease in expression over the two following time points, which was able to be attributed to the treatment effect of a lowered environmental pH. When the same experiment is analyzed looking at NHE3, there was an increase in protein expression over time with a significant increase of experimental values compared to controls at 1 and 2 hours, again attributed to the treatment effect of the lowered environmental pH. The H^+-ATPase is known to be very metabolically costly but also very efficient at lowering and maintaining systemic pH, using 3 ATP to transport 6-12 H^+ ions across the plasma membrane (Maxson & Grinstein, 2014). It is apparent that both H^+-ATPase and NHE3 are capable of functioning in this environment as the data show, and it can be suggested that the organism is limiting the energy used for acid-base regulation by switching to the use of the less efficient NHE3 in order to conserve energy

during an acidic episode. This is seen when looking at Figures 8 and 9, as there is a decrease in low sodium H^+-ATPase expression corresponding to an increase in NHE3 expression.

While the low sodium stream H^+-ATPase and NHE3 protein expression were the only results deemed statistically significant through the use of t-tests, the results of all other protein quantification experiments could be explained by differences in the water chemistry and ecological factors of their home environments. The data suggest that all samples in the high sodium streams have enough sodium for the NHE3 to function, rather than use the H^+-ATPase. It is known that the NHE3 is not as effective of a transporter at the H^+-ATPase at ridding the organism of H^+ very quickly. If the organism is not effectively removing acids from its body, it could begin to see the effects of acid stress, such as the altering of blood pH. This could be the reason there is a significant effect of treatment on blood pH in the ANCOVA results for the high sodium stream samples. As for the environmental effects, geographically, the high sodium stream (Road Prong) is on the Tennessee slope of the GRSMNP, and the low sodium stream (Beech Flats Prong) is on the North Carolina slope of the park. Streams are known to vary dramatically in water chemistry between subwatersheds, meaning differences in eastern and western facing slopes could be tremendous (Neff et al., 2013). As mentioned earlier, the surrounding geography can play a important role in the water chemistry and the acid neutralizing capabilities of surrounding terrain. The soil type, predominant vegetation type, and land use can all determine the extent to which atmospheric acid affects a stream, as well as time of year (Petty & Thorne, 2005; Deyton et al., 2009; Neff et al., 2013). All of these factors combined can completely change the way an environment functions, leading to differences in the way an organism

physiologically interacts with its environment. These changes could possibly lead to variances such as those seen between the fish from Beech Flats and Road Prong.

Pitfalls and Limitations

This study proposes a simple explanation to a very complex system, a limitation by time and scope of the project. The most important limitation when running these experiments is that true environmental conditions of an episodic stream acidification event will never be able to be replicated accurately in laboratory settings. What was have accomplished in this experiment is to show the effect of decreased pH on the Southern Appalachian Brook Trout in laboratory conditions. To test all of the effects of an episodic stream acidification event on the Southern Appalachian Brook Trout, a field-based study will have to be conducted with monitoring before and after the event.

Other pitfalls and limitations in research include the fact that conclusions were not completely validated, leaving it as a proposed explanation for the system. As mentioned before, the sodium content was not cooperative in the laboratory streams, resulting in both laboratory streams having slightly higher sodium contents than their corresponding field sites. This makes any statistical comparisons between the high and low sodium streams flawed, limiting the variations between high and low sodium streams to physiological differences based on native habitat of the animal. More time would also be needed to develop and run qRT-PCR on a full length NHE3 primer with the gill tissue cDNA synthesized in this experiment to quantify mRNA expression for the sodium alteration experiment, also giving more insight into the results of the protein quantification experiment.

Conclusions

The Southern Appalachian Brook Trout is constantly undergoing changes in its environmental pH due to atmospheric acid deposition affecting the acid balance of its native streams. These constant changes make it very difficult for these animals to maintain a systemic pH homeostasis, although it is necessary for their survival. A variety of methods are used to achieve this, ranging from bicarbonate buffers, non-bicarbonate buffers, and ion transport mechanisms. The most efficient means of maintaining an acid-base balance is through the use of ion transport mechanisms, specifically H^+-ATPase and NHE (Claiborne et al., 2002). H^+-ATPase and NHE3 were identified in the gills of the Southern Appalachian Brook Trout and quantified the expression of both mRNA and Protein expression in gill tissue. Furthermore, an explanation was proposed to describe how this system functions in laboratory conditions, in that the Southern Appalachian Brook Trout utilizes the metabolically costly but functionally efficient H^+-ATPase until the animal becomes low on energy at which time it switches to the more energy conserving but less functionally effective NHE3 to maintain homeostasis. This outcome was unexpected in experimentation and could lead to many further studies on the subject of pH and sodium balance linkage.

Future Directions

The building blocks of many future experiments have been set-up. More time will be needed to complete all aspects of experimentation laid out previously. Further experimentation is needed to determine fully if the animal is utilizing the H^+-ATPase or NHE3 depending on energy available, the results of this process could validate the results found in the current experiment. To determine if sodium affects the ability of H^+-ATPase or

NHE3 to function in the Southern Appalachian Brook Trout, the current experiment would have to be run again having better control of the sodium levels in the laboratory streams as well as a substantially higher number of samples and longer study periods. Seeing the difficulty in maintaining laboratory water chemistry after the fact, it would be wise to require any group asking for assistance from the National Parks Service to demonstrate the ability to maintain accurate laboratory conditions before collecting animals. Finally, analysis of the skin and kidney tissue samples taken during dissections could provide insight on the functions of H^+-ATPase and NHE3 in other parts of the Southern Appalachian Brook Trout.

NPS implications

When monitoring low sodium streams, it is important to assess energy levels of the organism. These streams will lack the sodium needed for NHE3 to function, forcing the fish to rely on the H^+-ATPase the maintain homeostasis. Using H^+-ATPase alone could severely deplete the animals' energy reserves, leaving the fish at risk to predation and decreasing the energy available for reproduction. Building off of this finding, it can be suggested from the data that there is a specific time where the animals become too energy depleted to continue using the H^+-ATPase and therefore switch to NHE3. This time frame presented in Figures 8 and 9 could be of use to the parks service when looking at the energy expenditure of these animals.

When monitoring high sodium streams, the acidosis of the fish seems to be of more concern than the exhaustion. Data suggest fish inhabiting higher sodium content streams may be more at risk of having an altered blood pH due to the lowered pH of the external environment. The lowering of an animal's blood pH can prove fatal in extreme cases.

The National Parks Service (NPS) staff located in the Great Smoky Mountains National Park graciously provided the animals used in this experimentation. This project coordinated with the NPS regularly throughout the experiment and has worked to tailor the results into management implications for the fisheries department. Data from this experiment as well as current literature were used to make these suggestions for the management of environmental acidification events in the park based on the presented findings. This research will hopefully lead to a better understanding of what is happening to the Southern Appalachian Brook Trout during episodic stream acidification events by the National Parks Service.

Images

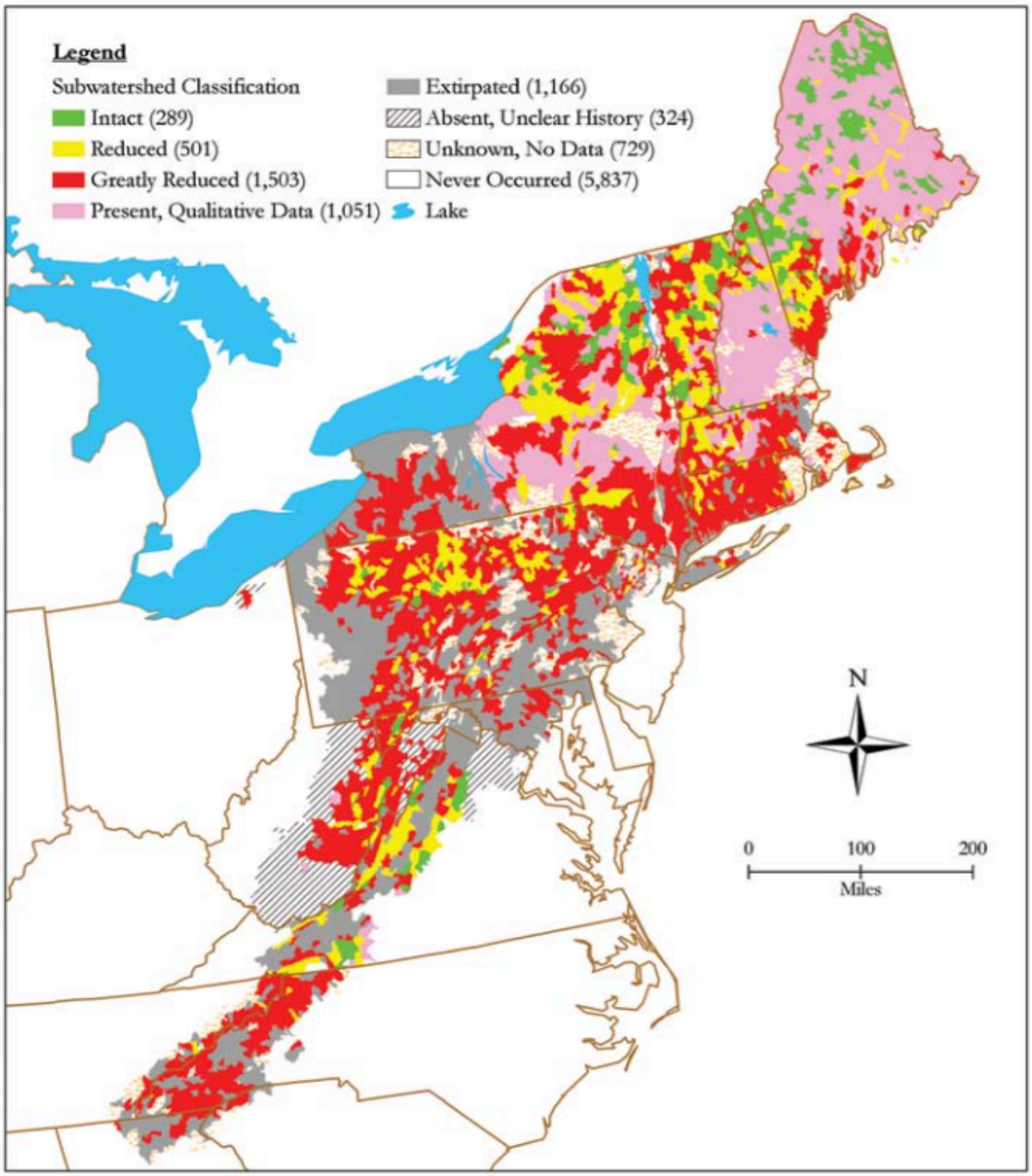

Image 1. Current range and relative loss of native Brook Trout over the last 100 years. All locations in the Great Smoky Mountains national park are reduced, greatly reduced, or extirpated. (Eastern Brook Trout Joint Venture, 2009)

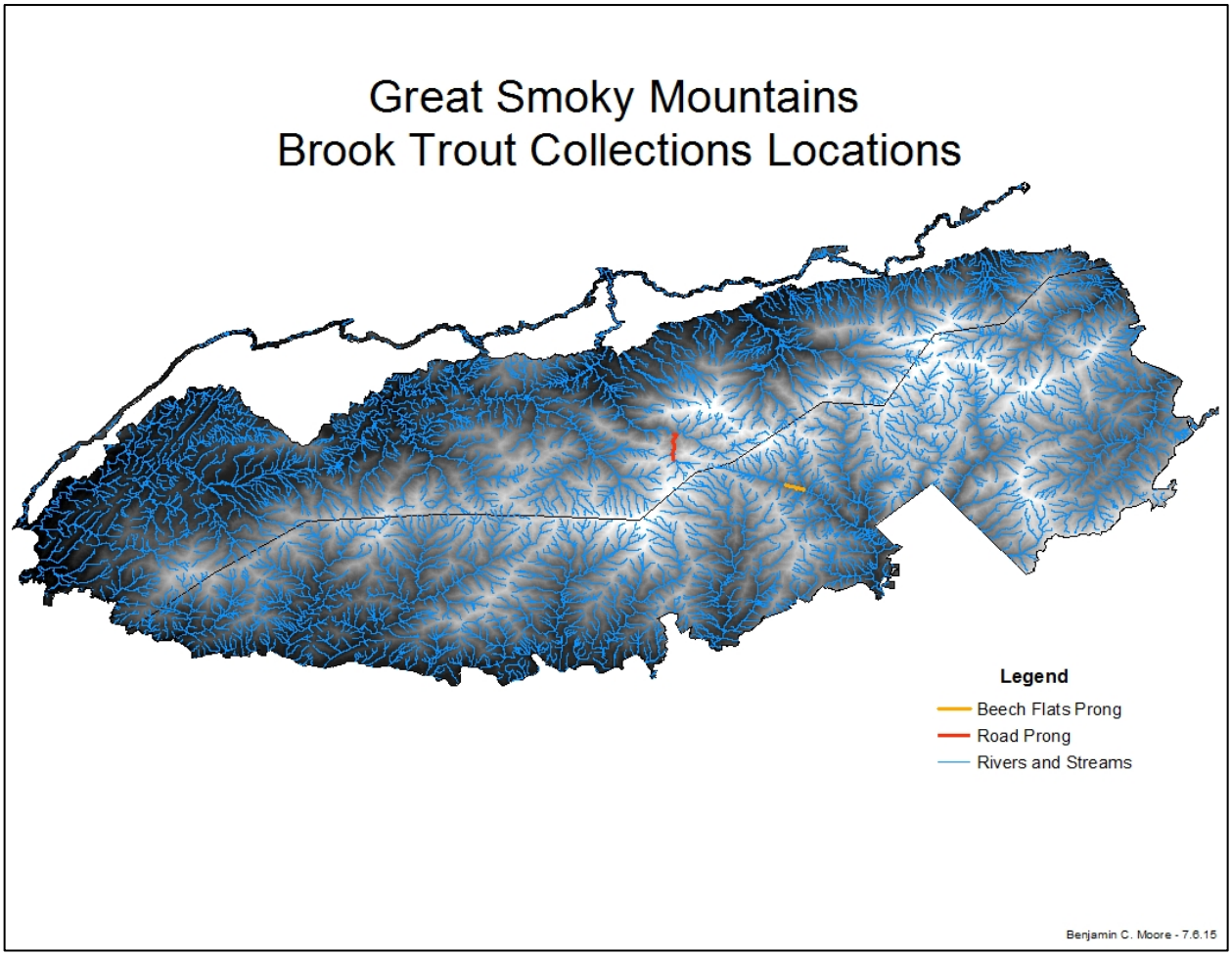

Image 2. Stream map of the Great Smoky Mountains National Park with collection streams highlighted. Road Prong is located on the Tennessee side of the park and was used for the constant sodium and sodium alteration experiment. Beech Flats Prong is located on the North Carolina Side of the park and was only used for the sodium alteration experiment.

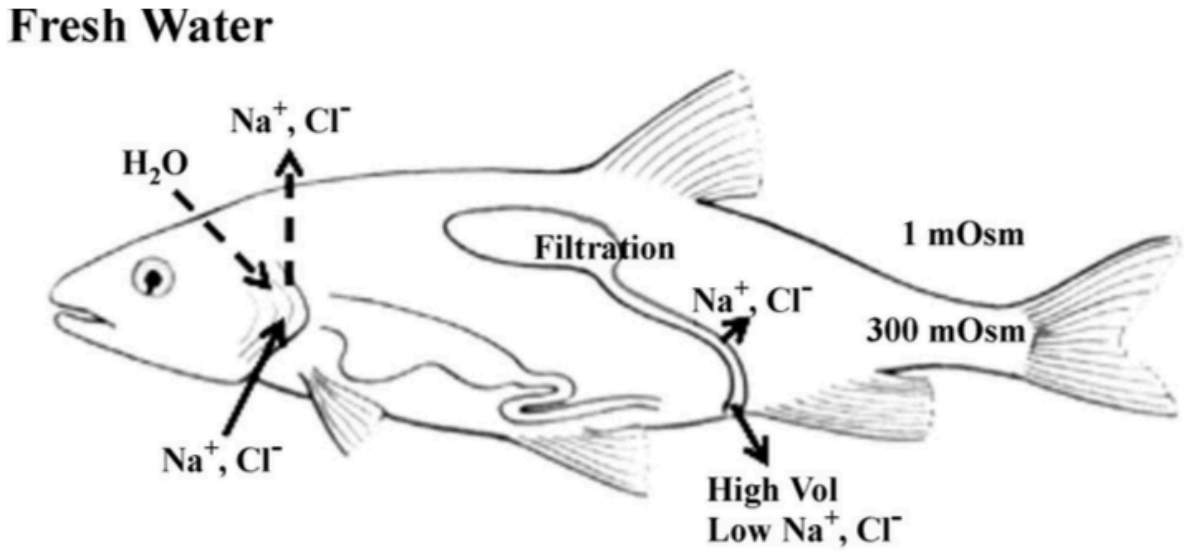

Image 3. Osmoregulation of a freshwater fish. The higher internal osmolarity of the freshwater fish compared to the external environment means the fish is constantly gaining water and losing salts (NaCl). To offset this loss the fish is constantly taking in salts from the environment and excreting large amounts of dilute urine. (Evans et al., 2008)

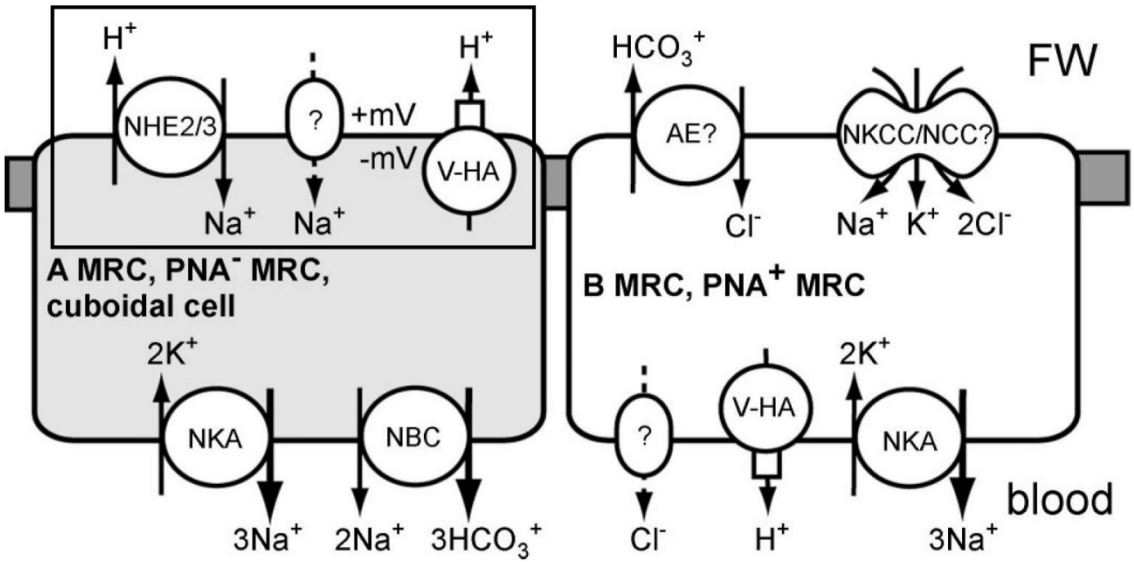

Image 4. Proposed mechanisms of ion transfer in the gills of freshwater teleost. The mechanisms of interest in this study are identified by the rectangular box on the α-MRC. In this image NHE3 is coupled with NHE2, the proposed location for ASIC is identified by the question mark and H+-ATPase is labeled V-HA. (Evans, 2008)

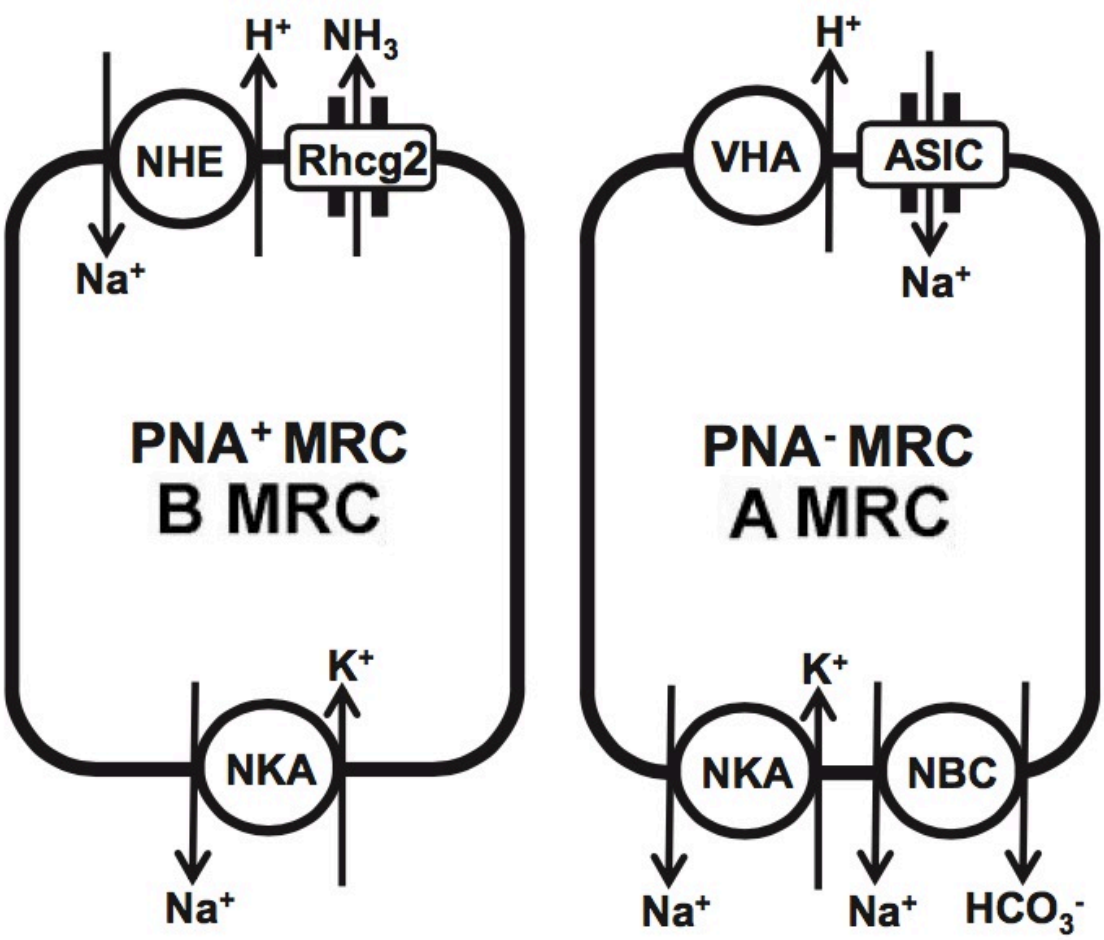

Image 5. Proposed working model of ASIC with H+-ATPase in the α-MRC of the freshwater teleost gill. ASIC functions on an inward sodium electrochemical gradient created by H+-ATPase function. (Edited from Dymowska et al., 2014)

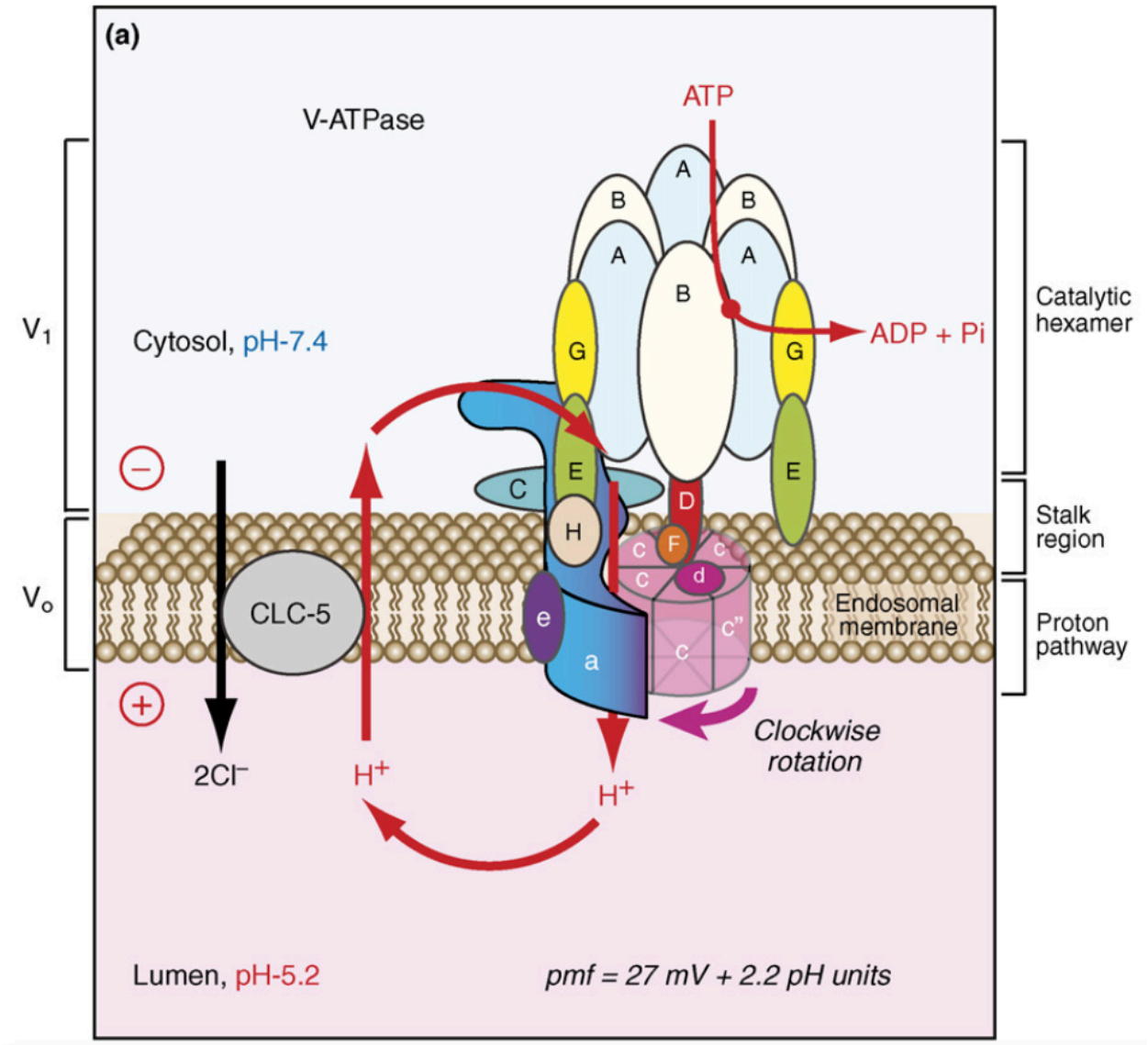

Image 6. Mechanical structure of H^+-ATPase. Structure consists of two domains, V_1 and V_0; V_1 is a rotary mechanism that completes the process of ATP-Hydrolysis. V_0 is responsible for transporting H^+ across the plasma membrane. (Marshansky and Futai, 2008)

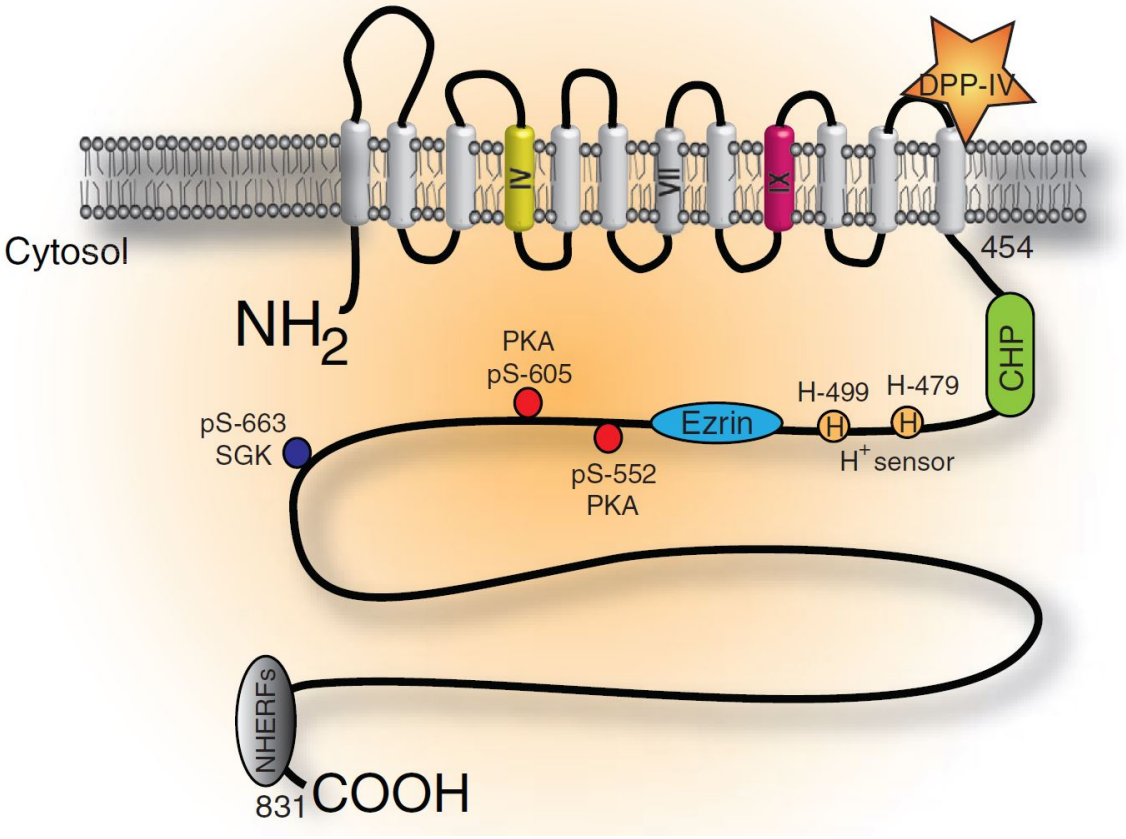

Image 7. Functional structure of NHE. Structure consists of two parts, N and C. N-terminus consist of 10-12 membrane spanning domains, and C-terminus consist of a cytocolic tail that is thought to play a regulatory role between NHE isoforms. (Alexander and Grinstein, 2009)

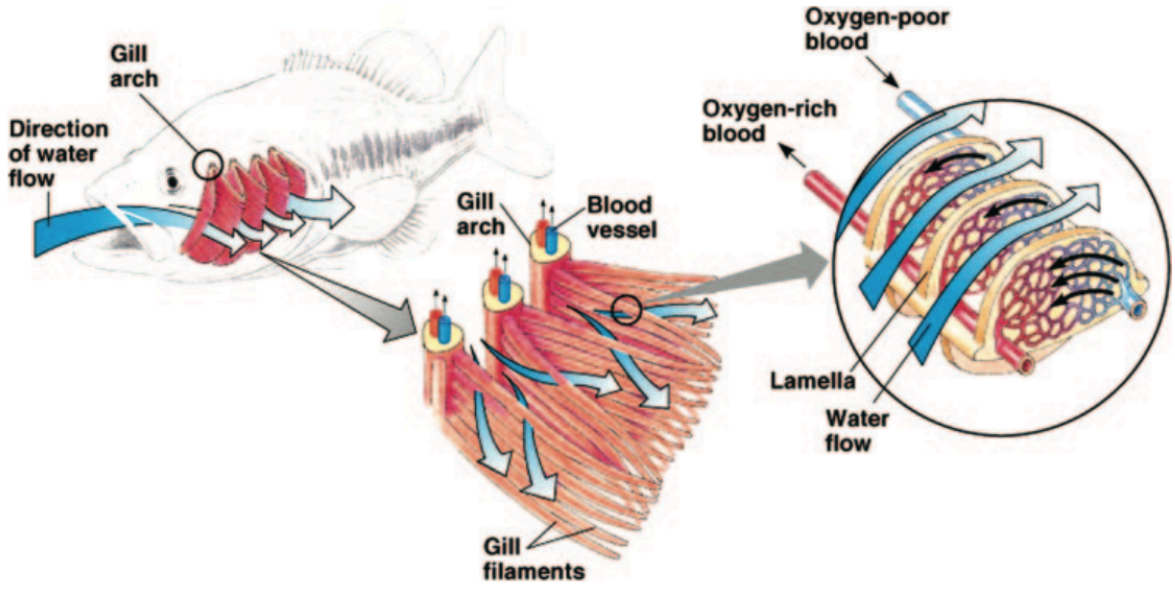

Image 8. Functional diagram of the fish gill. Water is allowed in through the mouth and let flow through the fish gill and out the operculum. Blood is cycled through the gill lamella countercurrent to water flow to allow for maximum potential diffusion of gas and ions across the gill epithelium. (Evans et al., 2005)

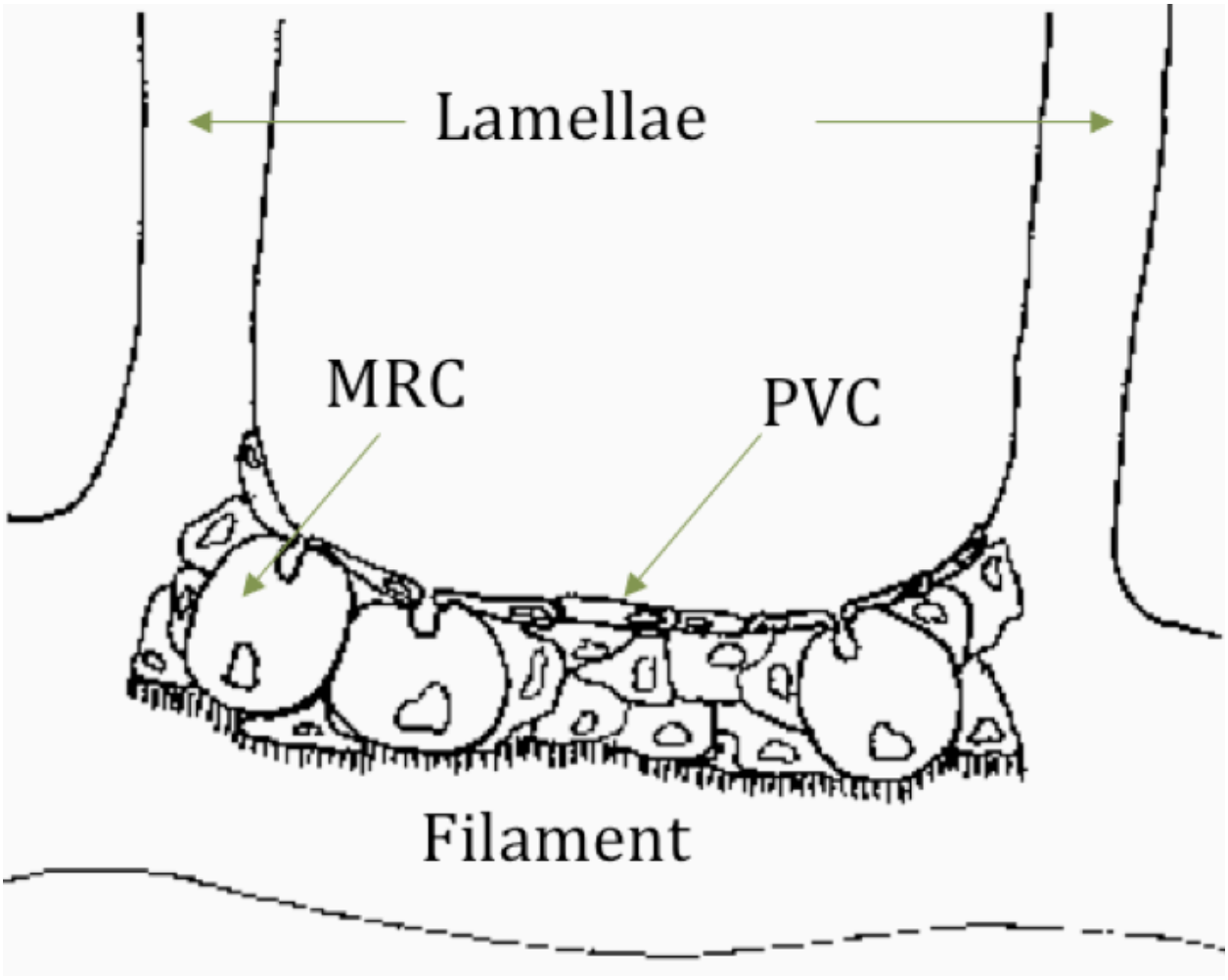

Image 9. Sketch of Interlamellar region of the fish gill. Pavement cells cover a large portion of the interlamellear region with MRC's scattered between. The gill lamellae are located on the gill filaments which can be seen in Figure 8. (Edwards, 2001)

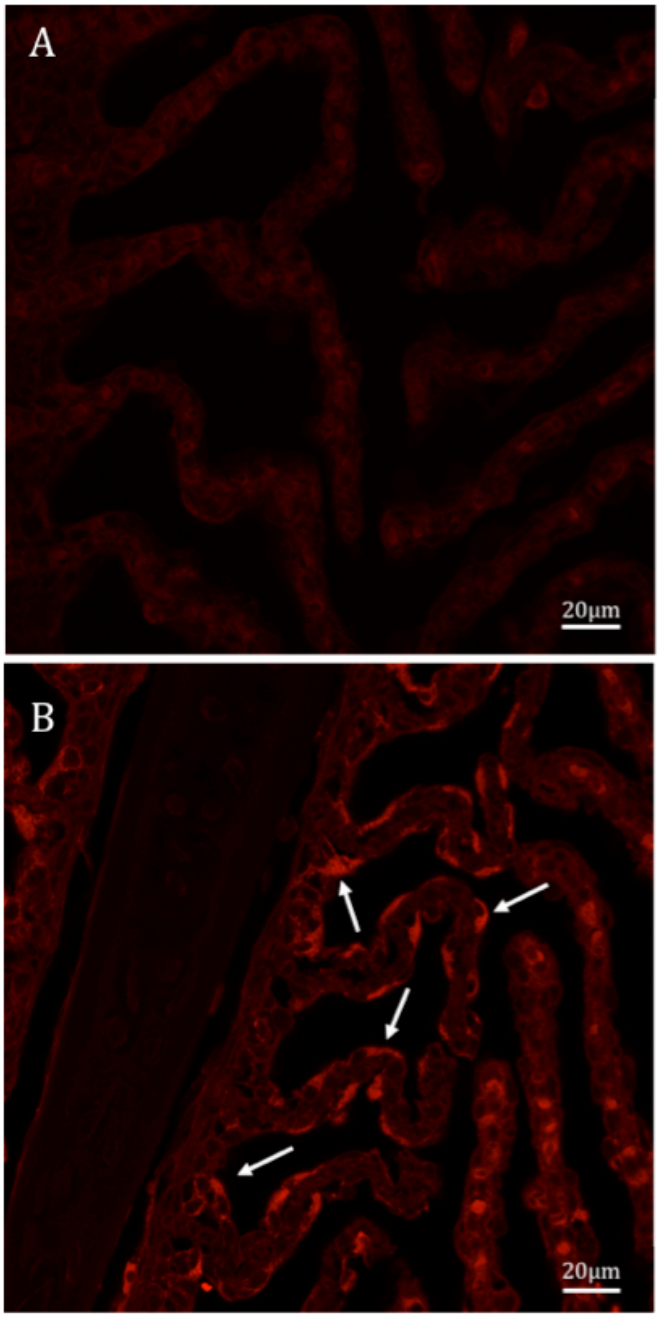

Image 10. Immunolocalization of H^+-ATPase in the Southern Brook Trout gill tissue. A) A negative control showing the absence of primary antibody staining. B) H^+-ATPase expression shown by fluorescence on the gill lamellae and interlamellar region. (Mikeworth, 2010)

Figures

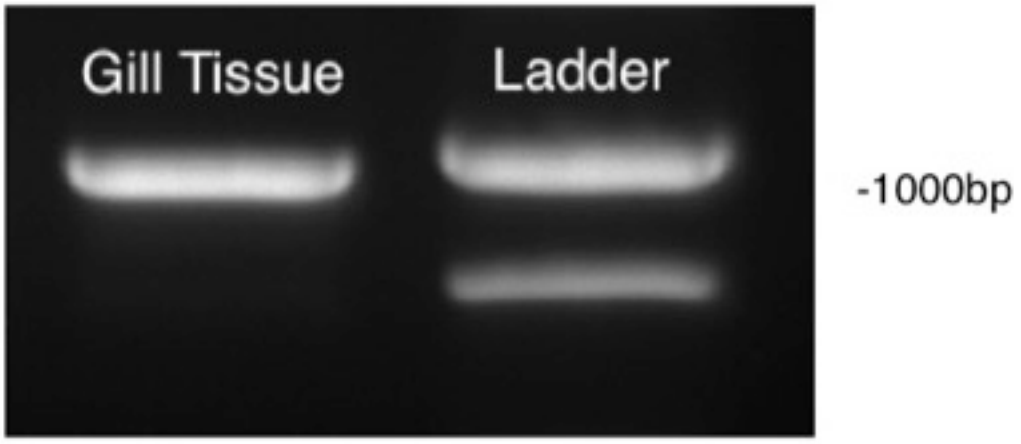

Figure 1. qPCR of Southern Brook Trout Gill Tissue run with the NHE3 primer. PCR product appears at ~1167bp as was reported by Ivanis et al. (2008).

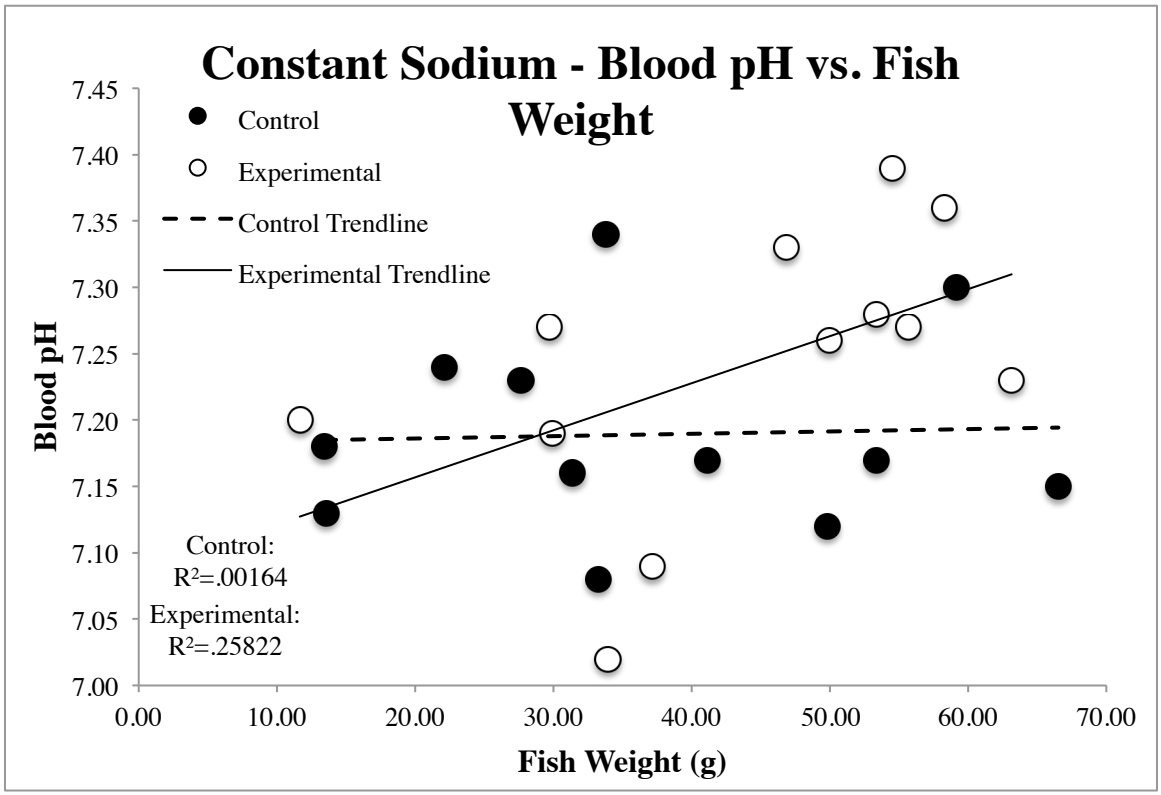

Figure 2. Post experimentation blood pH plotted against fish weight from the Constant Sodium Experiment. R^2 values for both treatment groups are shown in the bottom left.

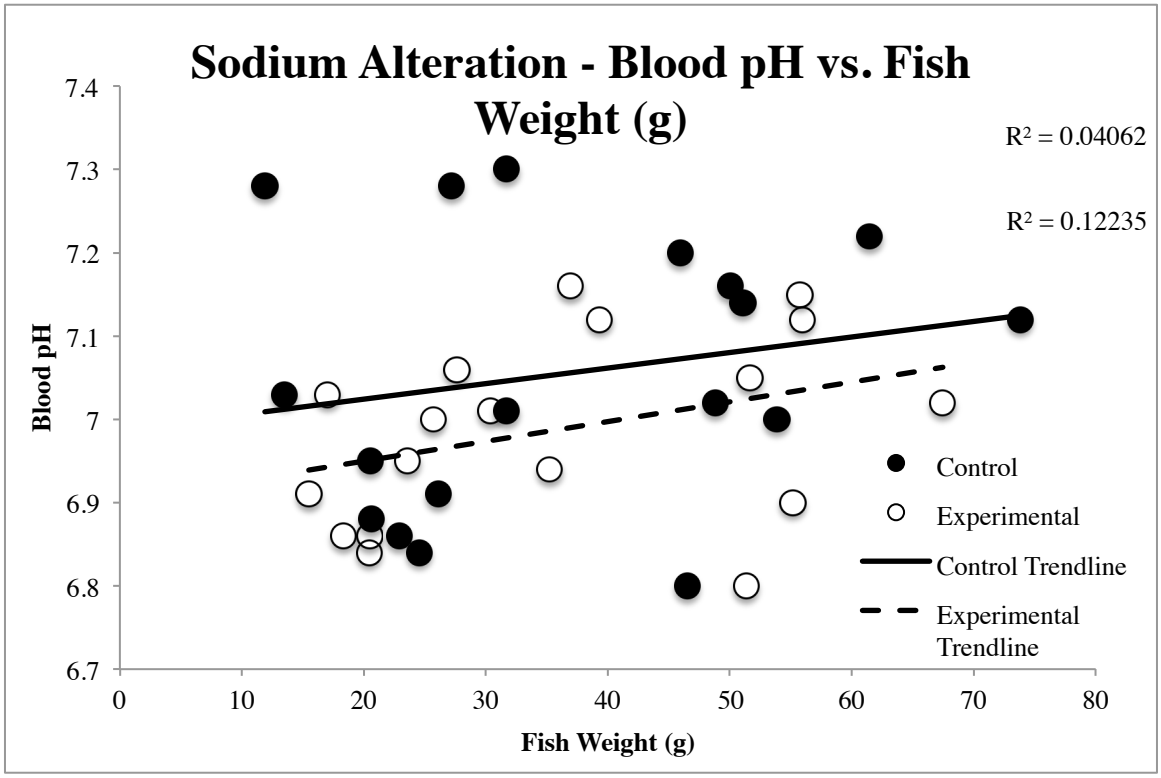

Figure 3. Post experimentation blood pH plotted against fish weight from the Sodium Alteration Experiment. R^2 values for both treatment groups are shown in the top right.

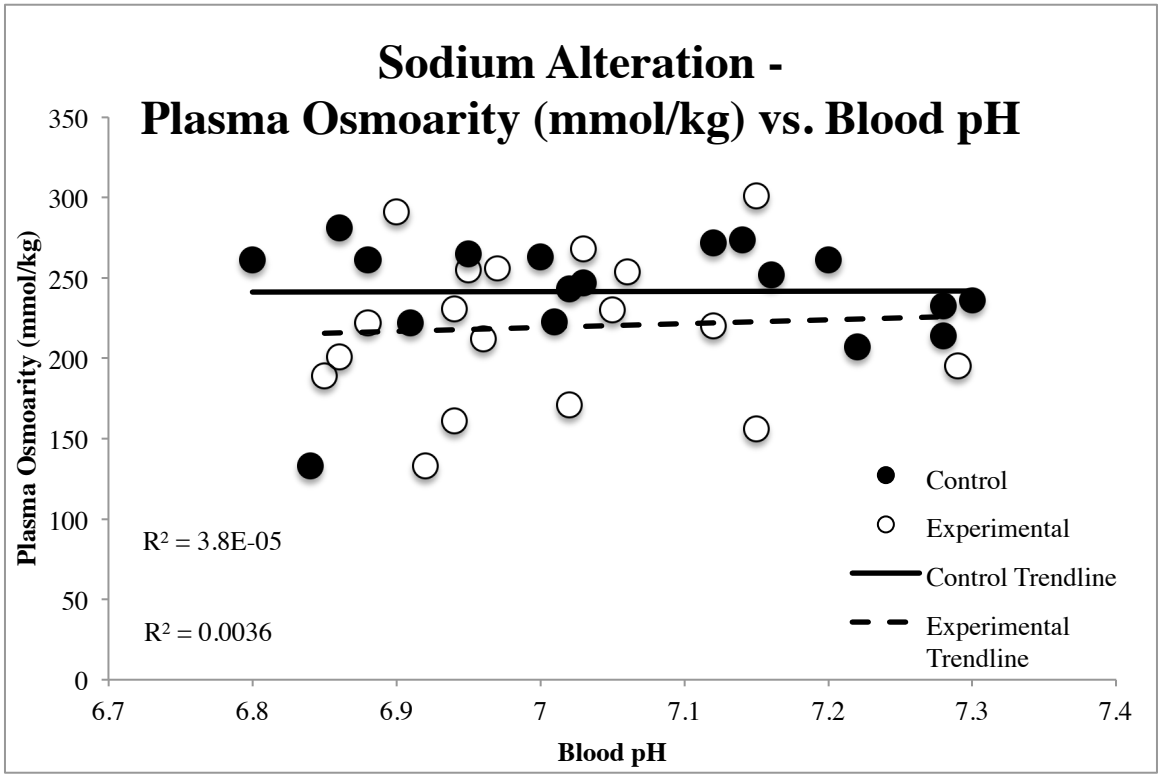

Figure 4. Post experimentation blood plasma osmolarity plotted against blood pH from the Sodium Alteration Experiment. R^2 values for both treatment groups are shown in the bottom left.

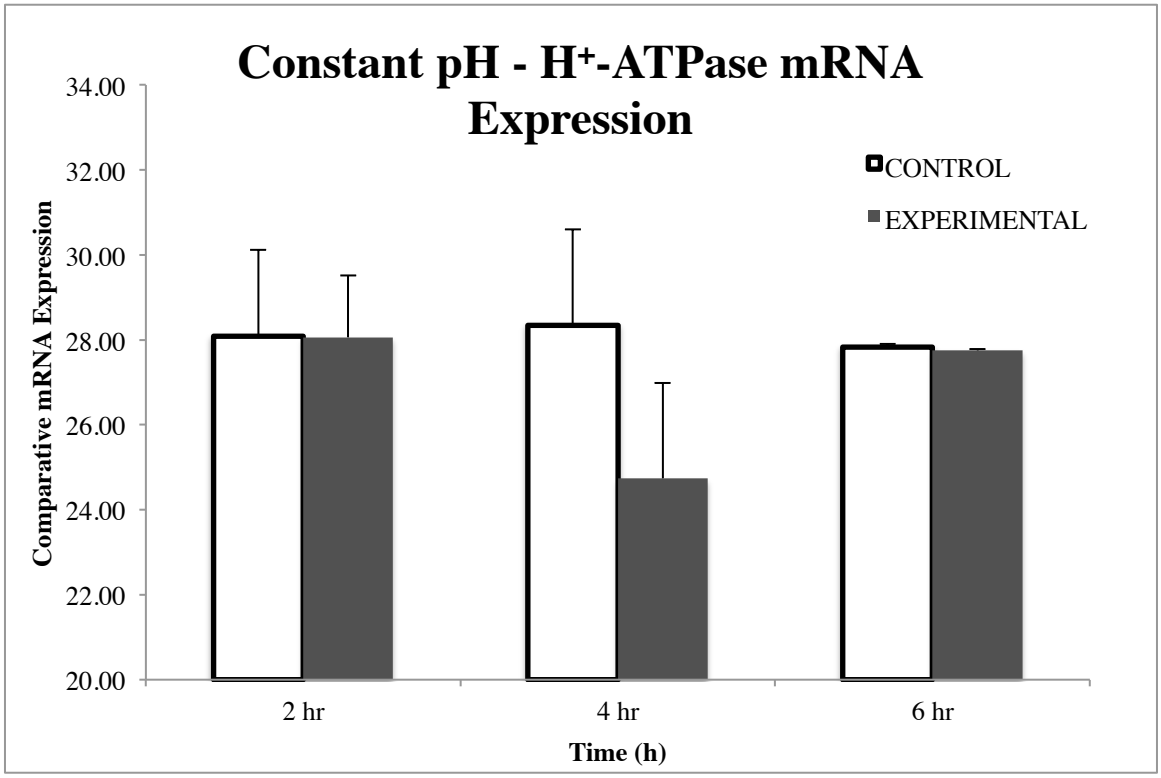

Figure 5. Relative H^+-ATPase mRNA expression for the constant sodium experiment. No significant changes were recorded between control and experimental treatments at any time point.

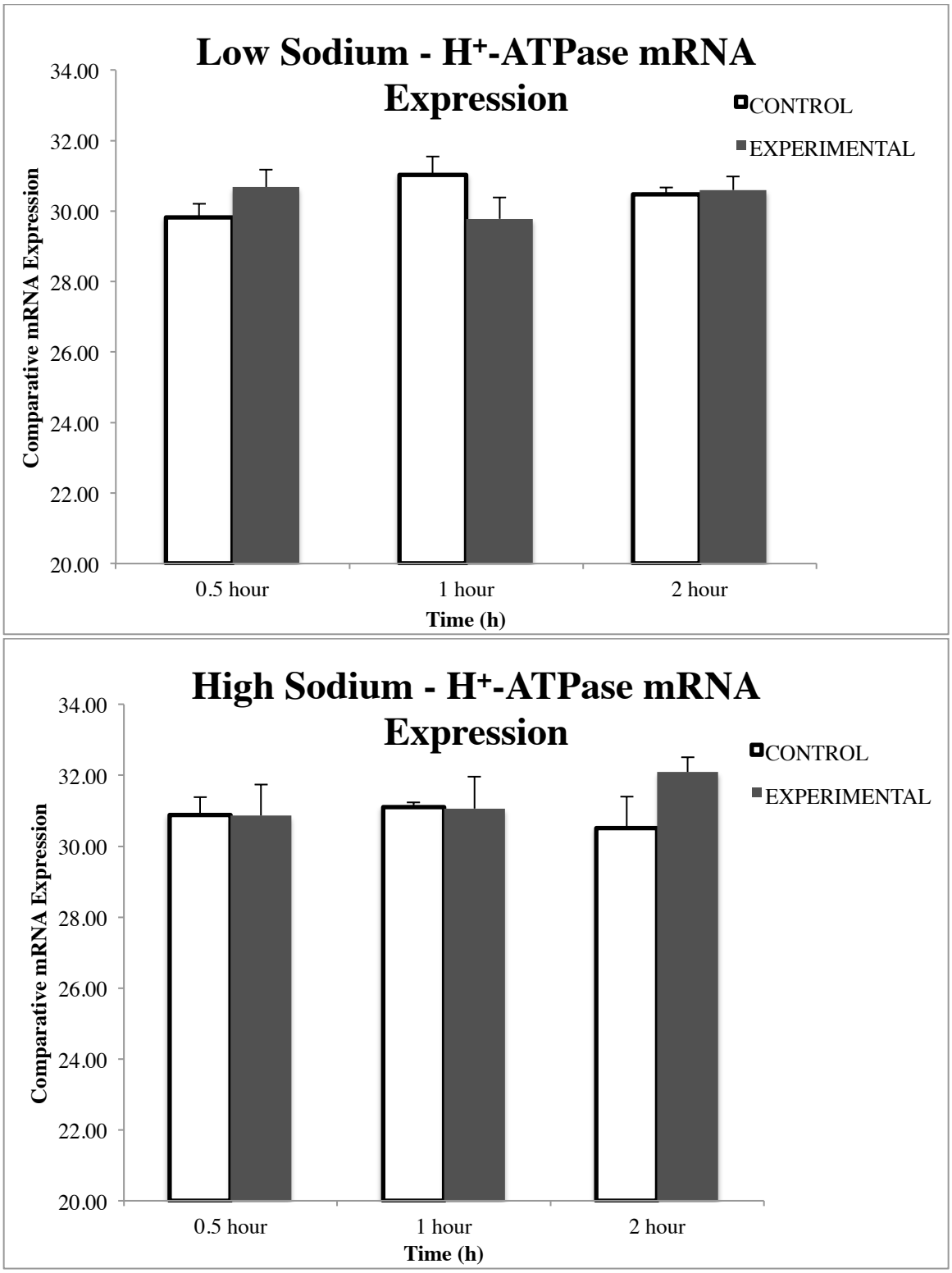

Figure 6. Relative H^+-ATPase mRNA expression for the sodium alteration experiment. No significant changes were seen for any sodium level, time point, or treatment.

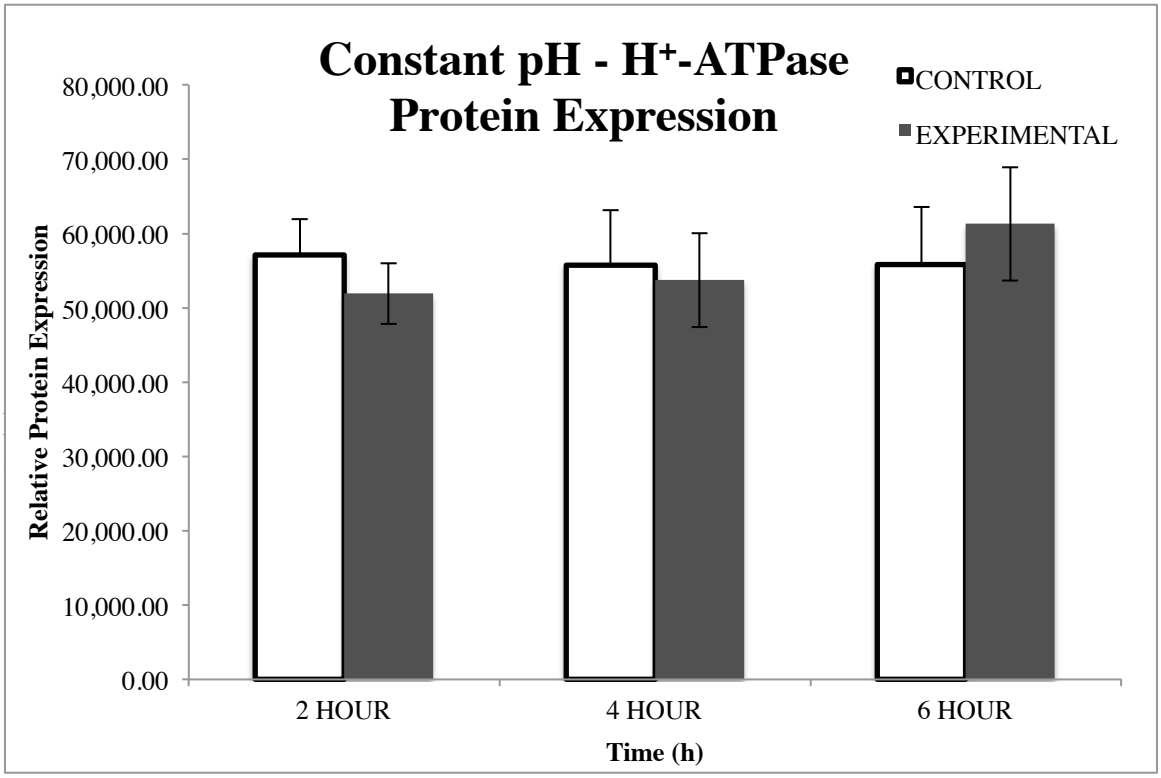

Figure 7. Relative H^+-ATP protein expression for the constant sodium experiment. No significant changes were noticed between control and experimental samples.

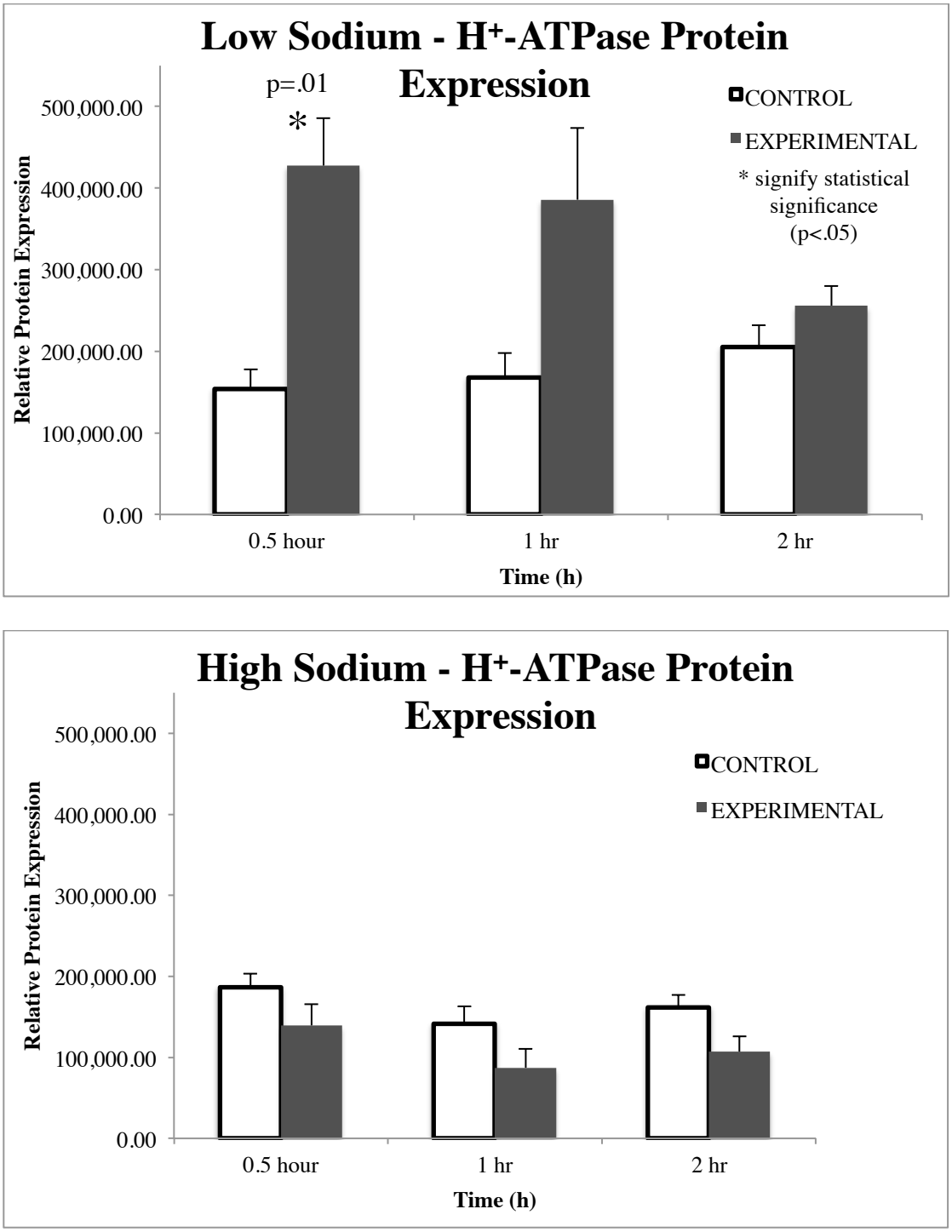

Figure 8. Relative H^+-ATPase protein expression for the sodium alteration experiment. Samples from the low sodium stream showed significant (p=.012) upregulation of H^+-ATPase protein expression at the 30-minute time point. Experimental samples from the low sodium stream also showed a nonsignificant trend of decreasing expression over time. Samples from the high sodium stream did not show any significant changes between control and experimental treatments.

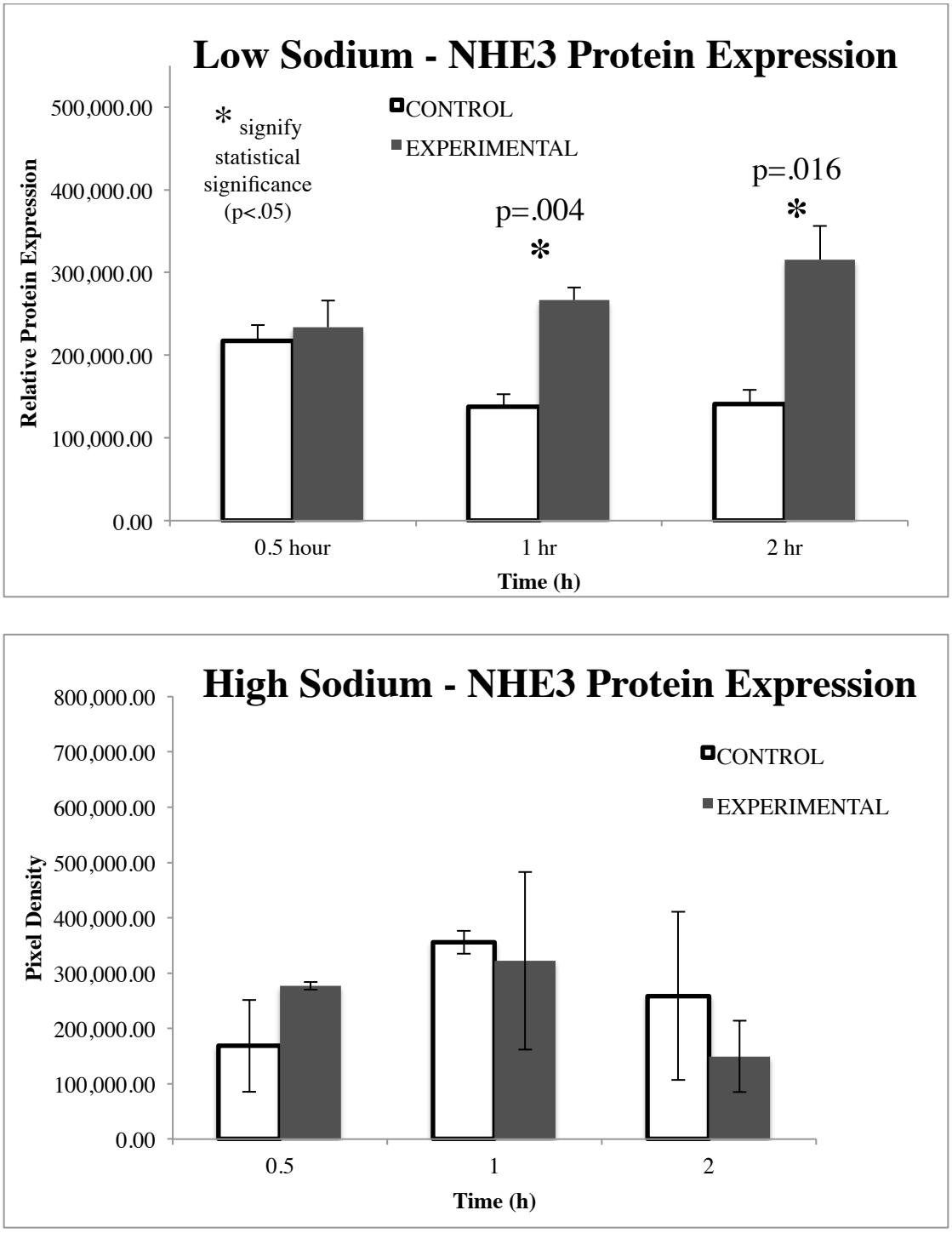

Figure 9: Relative NHE3 protein expression for the sodium alteration experiment. Samples from the low sodium stream showed significant upregulation of NHE3 protein expression in the experimental treatment at 1 hour (p=.004) and 2 hours (p=.016). Experimental treatments from the low sodium stream also showed a nonsignificant trend of increasing expression over time. Samples from the high sodium stream did not show any significant changes between control and experimental treatments.

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Vita

Benjamin Corbin Moore was born on September 29, 1991, to Dan and Tina Moore in Reidsville, North Carolina. Early in life, Benjamin discovered his love for the outdoors, roaming the family farm behind his house. His early interest in the environment led to a love of scouting and eventually an Eagle Scout Award. Benjamin graduated from Rockingham County High School in the spring of 2009. Following this graduation, he made his first move from Reidsville to Boone, where he attended Appalachian State University for his Bachelors of Science Degree. In the spring of 2013, Benjamin earned a Bachelors degree in Biology/Ecology, Evolution, and Environmental Biology. He was admitted to the Cratis D. Williams Graduate School at Appalachian State University in the fall of 2013 for a Masters degree in Biology. Under the guidance of Dr. Susan Edwards, Benjamin learned the skills of being a research biologist and graduated with his Masters degree in the summer of 2015.